Heat shock protein 90-sheltered overexpression of insulin-like growth factor 1 receptor contributes to malignancy of thymic epithelial tumors

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List of abbreviations:
Hsp90: heat shock protein 90
TEMs: thymic epithelial malignancies
Akt/PKB: protein kinase B
IGF-1R: insulin-like growth factor 1 receptor
CDK4: cyclin-dependent kinase 4
EGFR: epidermal growth factor receptor

Running title:
Relevance of Hsp90 and IGF-1R in thymic carcinomas
Abstract

Purpose: The underlying molecular mechanisms of thymic epithelial malignancies (TEMs) are poorly understood. Consequently, there is a lack of efficacious targeted therapies and patient prognosis remains dismal, particularly for advanced TEMs. We sought to investigate protumorigenic mechanism relevant to this understudied cancer.

Experimental Design: Recently established cell lines derived from thymic epithelial tumors were used as a model system. The anti-tumor activity of specific Hsp90 inhibitors was investigated by an analysis of cell viability, cell cycle, and apoptosis using MTT-assays and flow cytometry. Western blotting was used to investigate the altered expression of Hsp90 clients. Pharmacological inhibitors against select Hsp90 clients, as well as RNAi, were employed to test the relevance of each client independently. Tissue microarray analysis was performed to match the in vitro findings with observations obtained from patient-derived samples.

Results: Hsp90 inhibition significantly reduces cell viability of thymic carcinoma cells, induces cell cycle arrest and apoptosis, and blocks invasiveness. Hsp90 inhibition triggers the degradation of multiple oncogenic clients, e.g. IGF-1R, CDK4, and the inactivation of PI3K/Akt- and RAF/Erk-signaling. Mechanistically, the IGF/IGF-1R-signaling axis contributes to the establishment of the anti-apoptotic phenotype of thymic cancer cells. Finally, IGF-1R is overexpressed in advanced TEMs.

Conclusions: We have unraveled a novel protumorigenic mechanism in TEMs, namely Hsp90-capacitated overexpression of IGF-1R, which confers apoptosis evasion in malignant thymic epithelial cells. Our data indicate that Hsp90 inhibition, which simultaneously blocks multiple cancer hallmarks, represents a therapeutic strategy in TEMs that may merit evaluation in clinical trials.
Translational Relevance

Therapeutic approaches that improve the dismal situation of patients suffering from advanced and aggressive thymic epithelial malignancies (TEMs) are missing since the molecular mechanisms involved in the establishment of this cancer are largely unknown. Here we demonstrate, by using newly established in vitro models, that Hsp90 is a critical and multimodal modifier of TEMs. Pharmacological inhibition of Hsp90 elicits marked anti-tumor activity in thymic carcinoma cells and robustly inhibits multiple cancer-relevant signaling pathways. Along this line, we moreover show that the Hsp90 client IGF-1R is overexpressed in human thymic tumors and plays a role in establishing the malignant anti-apoptosis phenotype of thymic cancer cells. Collectively, these findings for the first time uncover molecular mechanisms that are vital to TEMs and that represent intervention points for targeted therapies. Our studies suggest that targeting Hsp90 or its client IGF-1R may warrant evaluation in clinical trials.
Introduction

Thymic epithelial malignancies (TEMs) show a broad spectrum of clinical and histological characteristics. According to WHO, TEMs are principally classified as type A, AB, B1, B2, and B3 thymoma. Finally, various subtypes of thymic carcinomas (formerly classified as type C thymomas) exist. Overall, type B3 thymomas and thymic carcinomas show a particularly unfavorable outcome (1, 2). Advanced disease is often irresectable and response rates to conventional chemotherapy are lower than 50% (3). Due to an inadequate knowledge regarding the oncogenic mechanisms of TEMs, very few targeted therapy approaches have been discussed, e.g. inhibition of the epidermal growth factor receptor (EGFR), or KIT. However, clinical trials have revealed that treatment with cetuximab or gefitinib (both targeting EGFR), as well as imatinib (targeting KIT) show only minimal activity in TEMs (3). Consequently, new therapeutic strategies are needed.

The hitherto disappointing results related to targeted therapies in this cancer might reflect the lack of representative model systems for preclinical studies, as well as the necessity to simultaneously block diverse oncogenic mechanisms in order to achieve potent anti-neoplastic activity in most tumors (4). Regarding the first aspect, adequate mouse models that recapitulate this cancer are still missing but advancements have been made by establishing stable cell lines derived from TEMs (5, 6). Regarding the second aspect, preliminary studies have shown beneficial effects of the multikinase inhibitors sorafenib and sunitinib in TEMs (7). Besides the use of nonspecific multikinase inhibitors, specific mono-targeted inhibition of the molecular chaperone Hsp90 represents a compelling strategy that allows a synchronized blockade of multiple malignancy driving mechanisms (8).
Hsp90 generally regulates the stabilization and activation of so-called “client” proteins, numerous of which are bona fide oncoproteins (9). Hence, Hsp90 controls signaling pathways involved in the establishment of all hallmarks of cancer (10). Hsp90 inhibition induces the simultaneous proteasomal degradation of various onco-clients. The therapeutic efficacy of Hsp90 inhibitors most likely relates to the concurrent shutdown of multiple pro-tumorigenic circuitries, hence preventing possible “escape” mechanisms of tumor cells by activating compensatory signaling pathways (10). Reports from clinical trials with the geldanamycin class of Hsp90 inhibitors (e.g. 17-AAG) demonstrate beneficial effects in several malignancies. However, this compound series has several limitations and an unfavorable toxicity profile (11, 12). With the advent of novel Hsp90 inhibitors that lack geldanamycin’s benzoquinone moiety, which is deemed accountable for most of the observed side effects, hope has been raised that these compounds will improve the clinical applicability of Hsp90 inhibition (13).

Here, we show that the Hsp90-sheltered activity of insulin-like growth factor 1 receptor (IGF-1R) contributes to the anti-apoptotic phenotype of thymic epithelial cancer cells. We demonstrate that IGF-1R overexpression manifests this newly identified mechanism of malignancy in advanced TEMs. Hsp90 inhibition reverses IGF-1R-mediated anti-apoptosis and induces multimodal anti-tumor activity. Our results suggest that Hsp90 inhibitors may represent promising therapeutic agents for the treatment of TEMs.
MATERIALS AND METHODS

Detailed methods are included as supplementary material.

Reagents

8-(6-iodobenzo[d][1,3]dioxol-5-ythio)-9-(3-(isopropylamino)propyl)-9H-purin-6-amine (PU-H71) was synthesized as previously reported (14). 17-allylamino-17-desmethoxygeldanamycin (17-AAG) and the EGFR inhibitor N-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine (gefitinib) were obtained from LC Laboratories (Woburn, MA, USA). The IGF-1R inhibitor cyclolignan picropodophyllin (PPP), the CDK4 inhibitor 2-Bromo-12,13-dihydro-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione, the Raf-1 inhibitor 5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl)methylene]-2-indolinone (Raf1 inhibitor I, GW5074), the Akt inhibitor 1,3-Dihydro-1-(1-(4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one (Akti-1/2, Akt inhibitor VIII), and the EGFR inhibitor 4-(3-Chloroanilino)-6,7-dimethoxyquinazoline (AG1478) were purchased from Calbiochem/Merck (Darmstadt, Germany). The selective IGF-1R inhibitor NVP-AEW541 (pyrrolo[2,3-d] pyrimidine derivative) and the dual IGF-1R/insulin receptor (IR) inhibitor BMS-536924 (1H-Benzimidazol-2-yl)-1H-pyridin-2-one) were from Selleck Chemicals (Houston, TX, USA). The broad-spectrum caspase inhibitor (Z-Val-Ala-Asp(OMe)-FMK) was from BioVision (Mountain View, CA, USA). The selective caspase-8 inhibitor (Z-IE(OMe)TD(OMe)-FMK)) and the selective caspase-9 inhibitor (Z-LE(OMe)HD(OMe)-FMK) were from Calbiochem/Merck. These compounds were dissolved in DMSO and stock solutions were stored at -20°C. Recombinant IGF-I and -II was from R&D Systems (Minneapolis, MN, USA) and the neutralizing anti-IGF-1R antibody (αIR3) was from Calbiochem. These compounds were dissolved in sterile...
PBS and stored as stock solutions at -20°C. Purified mouse IgG was from Millipore (Schwalbach, Germany).

Cell lines

The thymic carcinoma cell line, TC1889, and the thymoma cell line, T1682, were established, characterized and authenticated as previously described (5). Cells were cultured in RPMI-media containing HEPES (PAA Laboratories, Cölbe, Germany) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA) in an atmosphere containing 5% CO2. Cell lines were regularly checked for Mycoplasma infection using the VenorGEM® Mycoplasma Detection Kit (Minerva Biolabs, Berlin, Germany) according to the manufacturer's instructions.

Cell Viability Measurement

The number of viable cells was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide)-assay (EZ4U; Biomedica, Vienna, Austria), as previously described (5).

Cell Cycle Analysis and Detection of Hypodiploidy

DNA content was analyzed by flow cytometry of propidium iodide–stained nuclei using a FACSCalibur flowcytometer (Becton Dickinson, Franklin Lakes, USA) as previously described (5). Data were analyzed using CellQuest and ModFit LT 3.00 software.
Western Immunoblotting
Preparation of total protein lysates from human thymomas, thymic carcinomas, and normal thymic tissues was performed as previously described (15). Preparation of total protein lysates from cell cultures as well as subsequent SDS-PAGE and Western Immunoblotting was performed as previously described (16). See supplementary material for the antibodies employed.

Tissue microarray (TMA)
The tissue microarray was designed as previously described (15). Matching clinical data of the tissue array subjects including Masaoka stage (17) are given in Table 1.

Immunohistochemistry
Paraffin sections, each 4 μm thick, were cut, de-paraffinized in xylene and rehydrated with graded ethanol.
IGF-1R staining: 1 mM EDTA buffer (pH 9) was added to the slides and samples were heated by incubation in a microwave oven at 600 W for 3 x 5 min. After cooling, the slides were treated with hydrogen peroxidase in PBS to block the endogenous peroxidase. Nonspecific antibody-binding was blocked using the avidin/biotin blocking Kit according to the manufacturer’s instructions (Dako Cytomation, Glostrup, Denmark). Anti-IGF-1Rβ antibody (1:50, sc-713, Santa Cruz, Santa Cruz, USA) was applied and incubated in a moist chamber at 4°C overnight. Signal detection was performed with biotinylated secondary antibody in a 1:200 dilution (Dako Cytomation) followed by a streptavidin-biotin-complex and visualized with AEC-solution (Dako Cytomation) according to the manufacturer’s instructions.
IGF-I staining: Slides were subjected to 5 min heating at 120°C in Tris-EDTA-Buffer (pH 8.5) in a pressure cooker. Endogenous peroxidase activity was quenched by 5 min incubation with Peroxidase-Blocking solution (Dako REAL™, Dako Cytomation). Anti-IGF-I antibody (1:50, sc-7144, Santa Cruz) was applied for 30 min. Slides were subsequently incubated with the HRP-labeled Polymer anti-goat Histofine® (414161 F, Nichirei Cooperation, Tokyo, Japan) for 30 min and visualized using DAB solution (Dako Cytomation) according to the manufacturer’s instructions.

Generally, nuclear counterstaining was performed with Mayer’s hemalum. The sections were mounted with Kaiser’s glycerine gelatine. Adequate negative controls were obtained by replacing the primary antibody with antibody dilution buffer (Dako Cytomation). Non-epithelial compartments of thymi were excluded from the analyses and staining intensity was scored for each specimen on a scale of 0 to 3, in which 0 represents negative-, 1 low-, 2 medium -, and 3 high-staining intensity (see Figure 5) by two independent investigators (B.G. and R.R.; inter-observer agreement >0.7). In the cases of divergent scoring, a third observer (M.A.K.) decided upon the final category.

Statistical analysis

Statistical analyses of in vitro studies were performed with the two-sided student’s t-test. Statistically significant changes are given as p values: *p<0.05; **p≤0.01; ***p≤0.001. For TMA analyses, the differences in the frequency distribution of the intensity were analyzed using the Kruskal-Wallis test, with respect to stage and histological subtype. The Jonckheere-Terpstra test was employed to analyze trends. A difference was considered statistically significant if the p-value of the corresponding statistical test was less than 5% or equal (p≤0.05). Kappa statistics were used to
assess the degree of agreement between the ratings of the two observers. All statistical analyses were performed using the statistical software system SAS (SAS Institute, Cary, USA).
Results

Hsp90 inhibition induces multimodal anti-tumorigenic activity in thymic epithelial tumor cells

To start, the effect of the non-quinone Hsp90 inhibitor PU-H71 on cell viability of a thymic carcinoma cell line (TC1889) was investigated using MTT-assays. Hsp90 inhibition significantly reduced cell viability in a concentration- and time-dependent manner (Figure 1A). After 72 h, the concentration that achieved half maximal reduction of cell viability (IC50) was ≈0.25 μM. Treatment with PU-H71 induced G2/M arrest (Figure 1B), increased aneuploidy (data not shown) and resulted in cell death, as evidenced by increased hypodiploidy (>30% of cells in subG1) (Figure 1C). Cell death was attributable to apoptosis since PU-H71 treatment increased caspase-3 activity (data not shown) and induced a time-dependent cleavage of PARP (Figure 1D). Along these lines, broad-spectrum caspase inhibition significantly reduced PU-H71 treatment-induced caspase-3 activity (data not shown) and hypodiploidy (Figure 1E). Essentially the same results were obtained with the chemically distinctive Hsp90 inhibitor 17-AAG (supplementary Figure S1A-E), hence suggesting a common anti-tumorigenic activity of Hsp90 inhibition in TC1889 cells. Related to Hsp90 inhibition-induced apoptosis, we observed that inhibition of caspase-8 and -9 reduced hypodiploidy associated with PU-H71 treatment (Figure 1E). Additionally, PU-H71 treatment induced mitochondrial depolarization (Figure 1F), hence suggesting that Hsp90 inhibition unleashes intrinsic apoptosis in TC1889 cells.

Given that Hsp90 inhibition interferes with multiple mechanisms of malignancy (10), we investigated whether this strategy also blocks the cancer hallmark of tissue invasion. PU-H71 treatment inhibited the invasive capacity of TC1889 cells in a time- and concentration-dependent manner. A concentration of 1 μM was sufficient to
reduce invasion through Matrigel up to ~75% after 72 h as compared to controls (Figure 1G).

To further delineate the molecular basis for the observed anti-tumor activity of Hsp90 inhibitors in thymic cancer cells, we investigated the expression of well-established Hsp90 clients following Hsp90 inhibition. In TC1889 cells, PU-H71 treatment reduced the expression of EGFR, IGF-1R, cyclin dependent kinase 4 (CDK4), Akt, RAF-1, phosphorylated Akt, and phosphorylated Erk1/2 in a concentration- and time-dependent manner (Figure 1H). A marked reduction was achieved at PU-H71 concentrations >0.1 μM, which correlates well with the concentrations necessary to induce growth inhibition and apoptosis. In accordance with previous findings (16), expression of the inducible chaperone Hsp70 was increased. Essentially the same results were obtained with 17-AAG (supplementary Figure S1F), hence suggesting a common mechanism of action of Hsp90 inhibition. Likewise, PU-H71 treatment significantly reduced cell viability in the previously established thymoma cell line T1682, induced cell death as evidenced by increased hypodiploidy, and resulted in the simultaneous degradation and inactivation of aforementioned clients (supplementary Figure S2).

Collectively, Hsp90 inhibition exerts multi-modal anti-tumorigenic effects and simultaneously affects the expression and activity of multiple potential oncoproteins in thymic epithelial tumor cells.
The Hsp90 client IGF-1R represents a novel therapeutic target that contributes to apoptosis evasion in thymic cancer cells

Given the lack of insight into molecular mechanism relevant to TEMs, we attempted to dissect the contribution of each of the investigated Hsp90 clients to the establishment of the malignant phenotype of TC1889 cells.

We first employed pharmacological inhibitors to independently block the function of the clients EGFR, IGF-1R, CDK4, RAF-1, and Akt (supplementary Table S1). In order to corroborate our pharmacologically-based strategy and to take liabilities like off-target effects of the inhibitors into consideration, we further employed RNA interference (RNAi) to silence the highly druggable and hence clinically relevant clients IGF-1R, EGFR, and CDK4 (18-20) independently.

The different inhibitors employed reduced cell viability in a concentration- and time-dependent manner with the IGF-1R inhibitor PPP being the most potent compound (IC50: ~0.25 μM Figure 2A). Regarding EGFR inhibition, we also tested iressa/gefitinib, a drug previously tested in clinical trials with patients suffering from TEMs (3) and found that none of the gefitinib concentrations employed (up to 50 μM) were sufficient to achieve a half maximal reduction of cell viability (supplementary Figure S3). Since the efficacy of EGFR inhibitors can be associated with specific EGFR mutations (21), we tested whether these mutations can be found in TC1889 cells. We did not detect mutations in exons 18-21 of the EGFR gene (data not shown). In support of the results obtained by our pharmacological approach, RNAi experiments revealed that substantial siRNA-mediated depletion of the clients analyzed was associated with a significant reduction in cell viability. Again, effects
were strongest for IGF-1R knockdown with a ~40 % decrease in viability for both siRNAs after 120 h (Figure 2B).

Given that Hsp90 inhibition induced apoptosis in thymic carcinoma cells, we next investigated the contribution of Hsp90 clients to the cancer hallmark of apoptosis evasion. Therefore, we examined the apoptosis phenotype of TC1889 cells after treatment with inhibitors for each client at low and high concentrations. Among the compounds examined, only the IGF-1R inhibitor PPP potently induced apoptosis at low concentrations as evidenced by increased hypodiploidy (>25% of cells in subG1) and by PARP cleavage (Figure 2C and D). Additionally, IGF-1R inhibition with PPP induced cell death in a thymoma cell line, as indicated by increased hypodiploidy and cell viability reduction (supplementary Figure S4A and B).

In order to strengthen our results that suggested an important role of the Hsp90 client IGF-1R and its potential to represent a valid therapeutic target in TEMs, we additionally analyzed the effects of a neutralizing IGF-1R antibody (αIR3) and of the IGF-1R inhibitors NVP-AEW541 (NVP) and BMS-536924 (BMS). Notably, all of these strategies to interfere with IGF-1R signaling reduced TC1889 cell viability and significantly induced apoptosis (Figure 3A and B).

Related to IGF-1R inhibition-induced apoptosis, we also observed that inhibition of caspase-3, -8 and -9 reduced hypodiploidy associated with PPP treatment (Figure 3C). Additionally, PPP treatment induced mitochondrial depolarization (Figure 3D), suggesting that, similar to Hsp90 inhibition, IGF-1R inhibition also unleashes intrinsic apoptosis in TC1889 cells.

Together, these results suggest that among the Hsp90 clients investigated, IGF-1R contributes to the malignant phenotype of thymic epithelial tumor cells by its anti-
apoptotic properties. Given its druggability (22), IGF-1R might represent a potential therapeutic target in this cancer.

The IGF/IGF-1R signaling axis contributes to thymic cancer cell survival

In order to further investigate the relevance of IGF-1R-signaling in TEMs, we went on to analyze whether stimulation with IGF-I and –II affects cell viability. In TC1889 cells, IGF-I as well as IGF–II administration increased cell viability as compared to unstimulated controls. This effect was evident in cells grown in FCS-supplemented media but was considerably robust under serum-starvation conditions (Figure 4A). Correspondingly, IGF-I and –II administration significantly reduced cell death as a consequence of serum-starvation (Figure 4B), hence further suggesting a contribution of IGF-signaling to the anti-apoptotic phenotype.

A subsequent analyses of signal transduction pathways revealed that, under serum-starvation conditions, IGF-I administration induced the activation of Akt, GSK3β, MEK1/2, and Erk as evidenced by respective phosphorylations of conserved residues (Figure 4C). IGF-1R inhibition with PPP, αIR3, BMS, as well as NVP reduced the IGF-I-induced activation of PI3K/Akt- and MAPK/Erk-signaling. However, inhibitory effects, particularly on Akt phosphorylation (Figure 4C), were more marked with NVP and BMS which are both known to also inhibit the insulin receptor (22). Essentially the same results were obtained for IGF-I and -II stimulation under normal growth conditions with 10% FCS (supplementary Figure S5 and data not shown).

Surprisingly, in unstimulated non-starved TC1889 cells, PPP treatment for longer time periods (24-72 h) resulted in an increase in the activity of MAPK/Erk-signaling as indicated by augmented expression of phosphorylated Erk and MEK1/2, whereas Akt phosphorylation was only slightly decreased (Figure 4D). Essentially the same
results, although less pronounced, were obtained by inhibition of IGF-1R with the neutralizing antibody αIR3. Treatment with the IGF-1R/IR inhibitors NVP and BMS both induced a marked decrease in phosphorylated Akt, which corresponded to their aforementioned strong inhibitory effect on IGF-stimulated Akt signaling. Effects on MAPK/Erk signaling with these compounds were however heterogeneous, with BMS rather decreasing Erk phosphorylation, whereas NVP activated Erk (Figure 4D). Corresponding findings were obtained for 48 h compound treatment in serum-starved cells (supplementary Figure S6). Regarding IGF-1R inhibition with PPP, an activation of MAPK/Erk signaling was also detected following treatment with this drug in thymoma cells (supplementary Figure S4). Besides, we generally noticed that, in TC1889 cells, mono-targeted pharmacological inhibition of the Hsp90 clients EGFR, RAF-1, CDK4, and Akt resulted in differential activation of MAPK/Erk- and/or PI3K/Akt-signaling after 48 h and 72 h (supplementary Figure S7).

Taken together, these results confirm a growth-stimulating, anti-apoptotic property of the IGF-signaling axis in thymic cancer cells. Moreover, these findings demonstrate that IGF signaling is, at least partly, mediated via the PI3K/Akt- and MAPK/Erk-pathway. Lastly, our findings reveal that the IGF-1R inhibitors employed elicit varying effects on IGF-signaling.

**IGF-1R is overexpressed in thymic tumors**

Given the potential relevance of IGF-1R for the malignant anti-apoptotic phenotype of thymic epithelial tumor cells, we finally investigated whether these functional in vitro data are matched by expression data in human TEMs. Using Western immunoblot analyses, we initially found that IGF-1R expression in normal thymi samples was hardly detectable, whereas IGF-1R was expressed in the vast majority of TEMs

**CCR-10-1689_Revised_MS**
In order to corroborate and extend this finding and to take into account that the results may have been confounded by the presence of lymphocytic infiltrates, particularly in normal thymi samples, we performed immunohistochemistry employing tissue micro arrays (TMA) (Figure 5 and Table 1). Considering IGF-1R expression in the epithelial compartment only, a low expression intensity of IGF-1R was observed in normal thymi (Figure 5A, arrow). Overall, a trend was observed for higher IGF-1R expression in TEMs as compared to normal thymi (p=0.0005) and significant differences were observed between normal thymi and thymic tumors (p=0.006). In direct comparison to normal thymi, a higher expression of IGF-1R was observed in type B2 (p=0.032), and type B3 thymoma (p=0.008), as well as thymic carcinoma (p=0.008). Statistical analyses also revealed that there were significant differences regarding IGF-1R expression and Masaoka stages (p=0.044), with advanced stages showing higher IGF-1R expression (see supplementary Table S2 and S3). Additionally, an analysis of IGF-I expression (Figure 5B and Table 1) revealed a trend towards higher expression in thymic carcinoma (p=0.012), however no statistically significant differences could be calculated when normal thymi were compared with TEMs.

Taken together, IGF-I shows a trend for higher expression in TEMs and IGF-1R is significantly overexpressed in advanced TEMs, hence suggesting that IGF-1R contributes to malignancy of thymic tumors.
Discussion

Here, we dissected pro-tumorigenic mechanisms relevant to thymic epithelial malignancies (TEMs) and identified novel therapeutic targets. To our knowledge, this is the first functional analysis of factors relevant to TEMs that therefore differentiates itself from the descriptive nature of other studies, which are solely based on expression analyses by immunohistochemistry or global gene expression arrays, e.g. (23-25). Importantly, we provide evidence that the molecular chaperone Hsp90 plays a vital role in the maintenance of the cancer phenotype of thymic epithelial tumor cells. We demonstrate that Hsp90 “safeguards” the stability and activity of multiple molecules, such as IGF-1R, CDK4, and EGFR, which we found to differentially contribute to the survival of thymic cancer cells. In this regard, Hsp90 may rather support malignancy by capacitating various pro-tumorigenic aberrations (8), than act as a classical oncoprotein; a phenomenon referred to as “non-oncogene addiction” (26). Consequently, inhibition of Hsp90 interferes with multiple malignancy driving mechanisms and elicits multimodal anti-neoplastic activity (11). We show here that, in thymic cancer cells, this is reflected by the induction of cell cycle arrest and apoptosis, as well as the abrogation of invasiveness.

Given the observed anti-tumor potency of Hsp90 inhibition, alongside its known mechanism of onco-client degradation, we hypothesized that characterizing the pro-tumorigenic role of specific Hsp90 clients in thymic carcinoma cells might enable us to obtain insights into the largely unknown mechanisms of malignancy in this cancer. Using this biased approach, we show that IGF-1R contributes to the cancer hallmark of apoptosis evasion in thymic malignancies. Subsequent expression analyses in human thymic tumors moreover revealed that IGF-1R is overexpressed particularly in highly aggressive, advanced stage TEMs. Recently, TMA analyses in a large panel of
TEMs (n=132) confirmed an overexpression of IGF-1R and found correlations with WHO classification, stage and relapse (27). Interestingly, loss of heterozygosity in the 6q23.3-25.3 region, harboring amongst others IGF-2R, has been observed to be the most frequent chromosomal aberrations in TEMs (24). Absence of IGF-2R, which sequesters IGF, might theoretically lead to locally increased IGF concentrations (28). Moreover, comprehensive genomic analyses revealed that IGF2BP3/IMP-3, a translational activator of IGF mRNA (29), is highly overexpressed in thymic carcinomas (25). Our initial analyses revealed that, although a trend towards higher IGF-I expression in thymic carcinoma could be observed, IGF-I was not statistically significantly overrepresented in TEMs when compared to normal thymi. Nevertheless, support that IGF-signaling might represent a potential oncogenic mechanism in TEMs stems from transgenic mice overexpressing either IGF-II or the IGF-binding protein 4 (IGFBP4). Whereas thymic size is markedly increased in the former mouse models (30, 31), it is decreased in the latter, being associated with apoptosis (32). Consequently, the IGF-signaling axis that plays a relevant role in thymic epithelial cells under physiological conditions (33) might be exploited by an upregulation of IGF-1R in TEMs in order to manifest the malignant phenotype. In fact, prolonged disease stabilization was observed in a patient with metastatic thymoma in a phase 1 study of IGF-1R monoclonal antibodies (34). Additional experiments are necessary to analyze the pro-tumorigenic contribution of IGF-signaling in thymic tumors. Uncovering the effector mechanisms of IGF-1R-mediated signaling cascades that eventually contribute to apoptosis evasion in TEMs will very likely help in the identification of novel treatment strategies. Meanwhile, a phase 2 trial with an anti-IGF-1R antibody is currently being conducted in patients with TEMs (27).
Regarding other Hsp90 clients, our results support the notion that CDK4 contributes to thymic carcinoma cell survival. Correspondingly, previous reports have shown that inactivation of p16\(^{\text{INK4A}}\), which results in higher CDK4 activity (35), is involved in the progression of TEMs (36). Regarding EGFR, which is frequently overexpressed in TEMs (23), mutations that alter its susceptibility towards different inhibitors are rare in this cancer (37-39). Accordingly, we observed a rather poor performance of EGFR inhibitors in TC1889 cells, which do not harbor relevant EGFR mutations (data not shown). Further analyses are compulsory in order to reveal the actual importance of CDK4 and EGFR-signaling in the malignancy of TEMs.

Besides allowing an insight in pro-tumorigenic mechanisms of TEMs, our studies interestingly also revealed that mono-targeted inhibition of every onco-client alone, as exemplified by IGF-1R inhibition, can be associated with a prolonged activation of vital signaling cascades, e.g. MAPK/Erk-signaling. Off-target effects of the substances employed, as well as varying selectivity, e.g. the IGF-1R inhibitors NVP and BMS are known to also inhibit the insulin receptor (22), might contribute to these effects. Given that certain inhibitors elicit differential effects depending on the respective genetic background of cancer cells (40) care has to be taken in interpreting our results related to signaling pathways modified by the substances used. Additional studies are mandatory to comprehensively address this topic. Still, activation of potentially compensatory signaling pathways as a consequence of targeted inhibition of one factor could generally be related to the plasticity and adaptability of signaling networks (41). As previously demonstrated in other malignancies, these mechanisms can contribute to the occurrence of secondary resistance against single agent approaches (41-43). Further analyses are warranted to delineate whether compensatory pathway activation as a means of acquired resistance also relates to our initial observations.
So far, our attempts to develop TC1889 cells resistant to PPP have failed (data not shown).

Overall, a concurrent shutdown of multiple oncogenic mechanisms relevant to TEMs, including the parallel inhibition of PI3K/Akt- and MAPK/Erk-signaling has only been observed for Hsp90 inhibition, which was in contrast to all other single agent approaches tested here. Consequently, acquired resistance against Hsp90 inhibitors is unlikely to develop on the background of a dynamic switching between signaling circuitries. All currently known de novo or acquired resistance mechanisms of cancer cells against Hsp90 inhibitors relate to the chemical structure of geldanamycin analogs (44). Non-quinone Hsp90 inhibitors were not found to share this liability (45-47). The herein observed multimodal anti-tumor activity of the purine-scaffold Hsp90 inhibitor PU-H71, which has previously been reported to be well-tolerated in vivo (16, 48, 49) may provide a first rational for the initiation of clinical trials with novel Hsp90 inhibitors for individuals with advanced TEMs.

In summary, our approach allowed us to obtain valuable insights into pro-tumorigenic mechanisms involved in an inadequately understood cancer. Strikingly, the findings related to IGF-1R obtained in our in vitro model of thymic carcinomas could be matched by observations from patient-derived tumor samples hence underpinning the potential value of our thymic carcinoma cell line model. Until the development of animal models with which thymic epithelial tumorigenesis can be recapitulated, in vitro studies like the one described here might assist in the identification of potential therapeutic targets and treatment strategies for this peculiar cancer type.
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Figure 1: Hsp90 inhibition induces multimodal anti-tumor activity in thymic epithelial tumor cells

A) Cell viability reduction. TC1889 cells were incubated with PU-H71 and cell viability was analyzed using MTT-assays. Assays were performed in sextuple. Data are expressed as the mean ± SD (n≥3).

B) Induction of cell cycle arrest. TC1889 cells were incubated with PU-H71 and cell cycle distribution was assessed using FACS-analyses following PI-staining. Results of cells in G2/M are shown. Data are expressed as the mean ± SD (n=3).

C) Induction of cell death. TC1889 cells were incubated with PU-H71 and cell death (hypodiploid cells in subG1) was assessed using FACS-analyses following PI-staining. Data are expressed as the mean ± SD (n=3).

D) Induction of apoptosis. TC1889 cells were incubated with PU-H71 (1 μM) and PARP cleavage was investigated using Western immunoblotting. Representative results are shown (n≥3). Actin served as a loading control. fl.= full length; cl.= cleaved.

E) Caspase-dependence of apoptosis. TC1889 cells were incubated with PU-H71 and a broad caspase inhibitor (Casp̂i), a caspase-8 inhibitor (Casp8i) and a caspase-9 inhibitor (Casp9i) and cell death was assessed after 72 h using FACS-analyses following PI-staining. Data are expressed as the mean ± SD (n=3).

F) Mitochondrial depolarization. TC1889 cells were incubated with PU-H71 (1 μM) and mitochondrial membrane potential was analyzed after 48 h using FACS analysis following JC-1 staining. The uncoupler CCCP (100 μM) was used as a positive control inducing a marked shift in JC-1 fluorescence as compared to untreated controls (CO). Assays were performed in duplicate. Representative results are shown (n=3).

G) Inhibition of invasiveness. TC1889 cells were pretreated for 24 h with vehicle or PU-H71. Thereafter, viable cells able to migrate through Matrigel over a 48 h- and 72
h-period were quantified by phase contrast microscopy and data graphed. Data are expressed as the mean ± SD (n=3).

H) Simultaneous degradation and inactivation of potential oncoproteins.
Left) Concentration dependence. TC1889 cells were treated with vehicle or PU-H71 and the expression of the indicated proteins was investigated after 24 h using Western immunoblotting.
Right) Time dependence. TC1889 cells were treated with PU-H71 (1 μM) and the expression of the indicated proteins was investigated using Western immunoblotting. Representative results are shown (n≥3). Actin served as a loading control.

Figure 2: Hsp90 clients contribute to the establishment of the malignant phenotype of thymic carcinoma
A) Cell viability reduction. TC1889 cells were treated with pharmacological inhibitors against the Hsp90 clients indicated and cell viability was assessed using MTT-assays. Assays were performed in sextuple. Data are expressed as the mean ± SD (n≥3).
B) RNAi. TC1889 cells were transfected with the indicated siRNAs against the select Hsp90 clients and the expression of respective proteins was investigated using Western immunoblotting. Representative results are shown (n=3). Actin served as a loading control. Cell viability following transfection with respective siRNAs was measured using MTT-assays. Assays were performed in sextuple and data are expressed as the mean ± SD (n=3).
C) Induction of cell death. TC1889 cells were treated with pharmacological inhibitors against select Hsp90 clients and cell death was evaluated by FACS-analyses following PI-staining. Data are expressed as the mean ± SD (n=3).
D) Induction of apoptosis. TC1889 cells were treated with vehicle or the indicated pharmacological inhibitors against select Hsp90 clients and PARP cleavage was analyzed by Western Immunoblotting. Representative results are shown (n=3). Actin served as a loading control. fl.= full length; cl.= cleaved.

**Figure 3: IGF-1R contributes to thymic cancer cell survival**

A) Cell viability reduction. TC1889 cells were treated with pharmacological inhibitors against IGF-1R (NVP and BMS), as well as a neutralizing IGF-1R antibody (αIR3) and cell viability was assessed using MTT-assays. Mouse IgG1 antibody was employed as a reference control for the effects of αIR3 at respective concentrations. Assays were performed in sextuple. Data are expressed as the mean ± SD (n≥3).

B) Induction of cell death. TC1889 cells were treated with pharmacological inhibitors against IGF-1R as well as a neutralizing IGF-1R antibody and cell death was evaluated by FACS-analyses following PI-staining. Data are expressed as the mean ± SD (n=3).

C) Caspase-dependence of apoptosis. TC1889 cells were incubated with the IGF-1R inhibitor PPP and a broad caspase inhibitor, a caspase-8 and a caspase-9 inhibitor and apoptosis was assessed after 72 h using FACS-analyses following PI-staining. Data are expressed as the mean ± SD (n=3).

D) Mitochondrial depolarization. TC1889 cells were incubated with PPP (1 μM) and mitochondrial membrane potential was analyzed after 48 h using FACS analysis following JC-1 staining. The uncoupler CCCP (100 μM) was used as a positive control. Assays were performed in duplicate. Representative results are shown (n=3).
Figure 4: Relevance of the IGF/IGF-1R signaling axis in thymic carcinoma cells

A) Cell viability increase. TC1889 cells were treated with IGF-I or -II under normal growth conditions (10% FCS) as well as serum-starvation conditions (0% FCS) and cell viability was assessed using MTT-assays. Assays were performed in sextuple. Data are expressed as the mean ± SD (n=3).

B) Apoptosis rescue. TC1889 cells were treated with vehicle, IGF-I or –II (100 ng/ml) for 72 h under serum-starvation conditions. Cell death was evaluated by FACS-analyses following PI-staining. Data are expressed as the mean ± SD (n=2).

C) TC1889 cells were serum-starved for 16 h, treated with vehicle or the IGF-1R inhibitor PPP, NVP, BMS or the neutralizing IGF1R antibody αIR3 for 2 h, and then stimulated with IGF-I (100 ng/ml) for 15 min. The activity of PI3K/Akt- and MAPK/Erk-signaling was investigated by an analysis of the expression of phosphorylated Akt, GSK3β, MEK1/2, and Erk using Western immunoblotting. Representative results are shown (n=3). Actin served as a loading control.

D) TC1889 cells were treated with vehicle or PPP, NVP, BMS or αIR3. The activity of PI3K/Akt- and MAPK/Erk-signaling was investigated as mentioned above. Representative results are shown (n=3). Actin served as a loading control.

Figure 5: The Hsp90 client IGF-R1 is overexpressed in thymic tumors

IGF-1R and IGF-I protein expression in normal thymus, different thymoma subtypes and thymic carcinomas evaluated by immunohistochemistry. Clinical data of each specimen and respective stagings are given in Table I.

A) IGF-1R: Normal thymus (case no.38, score: 1); thymomas: type A (case no.8, score: 2), type AB (case no.12, score: 1), type B1 (case no.20, score: 2), type B2
(case no.23, score: 2), type B3 (case no.30, score: 3); thymic carcinoma (case no.36, score: 3).

B) IGF-I: Normal thymus (case no.38, score: 1); thymomas: type A (case no.8, score: 1), type AB (case no.14, score: 1), type B1 (case no.16, score: 2), type B2 (case no.23, score: 1), type B3 (case no.31, score: 3); thymic carcinoma (case no.34, score: 2).
Figure 2
Figure 3
Figure 4
Figure 5
Table 1  Expression patterns for IGF-1R and IGF-I and additional clinical data.

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<th>Staging (Masaoka)</th>
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* Sex: M = male; F = female;

Representative expression intensities for IGF-1R and IGF-I are depicted in Figure 5A and 5B, respectively.
Heat shock protein 90-sheltered overexpression of insulin-like growth factor 1 receptor contributes to malignancy of thymic epithelial tumors

Marco Breinig, Philipp Mayer, Andreas Harjung, et al.

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