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INVESTIGATIONS ON THE ROLE OF HSP90 IN THE PATHOGENIC GLUCOCORTICOID RESISTANCE OF CORTICOTROPH PITUITARY ADENOMAS

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ABBREVIATIONS

^{3}H–Dex	tritiated Dexamethasone
AAA	P548A/T549A/V551A mutant of rat GR
ACTH	Adrenocorticotropin
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
Cdc2	Cell division cycle protein 2 homolog
CTD	C–terminal domain of Hsp90
DAB	3,3'–Diaminobenzidine
DAPI	4,6–Diamidino–2–phenylindole
DDW	Double distilled water
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
Gc	Glucocorticoid
GR	Glucocorticoid receptor
H_2O_2	Hydrogen peroxide
HEPES	2–(4–(Hydroxyethyl)–1–piperazinyl)–ethanolsulforic acid
Hsp90	Heat shock protein 90
ICC	Immunocytochemistry
IHC	Immunohistochemistry
kD	Kilodalton (molecular weight)
MD	Middle domain of Hsp90
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NP–40	Nonidet P–40
NTD	N–terminal domain of Hsp90
ON	Over night
ONPG	o–Nitrophenyl–β–D–galactopyranosid
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
PVDF	Polyvinylidene fluoride
RT	room temperature
SDS	Sodium dodecyl sulphate
SDS–PAGE	SDS polyacrylamid gel electrophoresis
TEMED	Tetramethylethylenediamine
TBS	Tris buffered saline
TBST	TBS with 0.1% Tween–20
WT	Wild type

1 SUMMARY

The main function of glucocorticoids in corticotroph cells is to suppress proopiomelanocortin, the precursor of the stress hormone adrenocorticotropin (ACTH). Cushing's disease is a rare but severe neuroendocrine condition caused by partially glucocorticoid resistant corticotroph adenomas, which consequently secrete excessive amounts of ACTH in an uncontrolled fashion. The patients suffer from chronic hypercortisolism due to excessive stimulation of the adrenal glands by ACTH to produce glucocorticoids. Impairing mutations of the glucocorticoid receptor (GR) only sporadically explain the reduced glucocorticoid sensitivity in the adenomas – the molecular mechanism behind the partial resistance is poorly understood.

The function of GR depends on direct interactions with the molecular chaperone Hsp90. Both the reduction and overexpression of Hsp90 impedes GR activity in different experimental settings. Therefore, the expression of the inducible Hsp90α isoform was determined in biopsy specimens of corticotroph pituitary adenomas from patients with Cushing's disease. Its strong overexpression compared to normal human pituitary cells paved the way to study its role in the function of corticotroph adenomas using small molecules which target Hsp90.

The three distinct Hsp90 inhibitors 17–AAG, Novobiocin and Silibinin showed antiproliferative effects in AtT–20 cells through the degradation of the oncogenic client kinase Cdc2, a hallmark of pharmacologic inhibition of Hsp90. Surprisingly, only the N–terminal Hsp90 inhibitor 17–AAG caused the degradation of GR, as was reported also for other Geldanamycin–based Hsp90 inhibitors. Neither Silibinin nor the C–terminal Hsp90 inhibitor Novobiocin affected GR protein levels. These converging effects led to the assumption that both compounds bind to the same domain in Hsp90. It was shown here that Novobiocin displaces Silibinin from the C–terminal domain of Hsp90, and that these compounds dissociate mature GR from Hsp90 at the biochemical level. As a result, increased levels of mature receptor were present in the cell able to bind glucocorticoids with high affinity. This novel molecular mechanism proved to potentiate GR transcriptional activity in AtT–20 cells. The potentiation in GR activity also led to enhanced suppression of ACTH elicited by low concentrations of Dexamethasone in AtT–20 cells and in primary cultures of human corticotroph adenomas from patients with Cushing's disease. In contrast, Silibinin did not show effects on rat normal pituitary cells. Finally, Silibinin reduced tumor growth, partially reverted hormonal alterations, and alleviated symptoms in a mouse allograft model for Cushing's disease.

These results suggest that the regulation of GR sensitivity by overexpressed Hsp90 may represent a pharmacologically reversible mechanism in the pathogenesis of this disease. Together, a proof of principle is provided that the clinically safe Hsp90 inhibitor Silibinin potentially restores glucocorticoid sensitivity in corticotroph adenomas *in vitro* and *in vivo*, and that it might be used to treat Cushing's patients in the future.

2 ZUSAMMENFASSUNG

Die zentrale Funktion von Glukokortikoiden in kortikotrophen Zellen ist die Supprimierung von Proopiomelanokortin, dem Vorgänger des Stresshormons Adrenokortikotropin. Morbus Cushing ist eine seltene neuroendokrinologische Erkrankung, die durch partiell Glukokortikoid–resistente kortikotrophe Adenome der Adenohypophyse verusacht werden. Die exzessive Produktion und Sekretion von Adrenokortikotropin führt zu exzessiver Stimulation der Nebenierenrinde zur Produktion von Glukokortikoiden, und die Patienten leiden unter chronischem Hyperkortisolismus. Mutationen in dem Gen, das für den Glukokortikoidrezeptor (GR) codiert, können die Glukokortikoidresistenz nur in seltenen Fällen erklären – der molekulare Mechanismus, der zu der partiellen Resistenz führt, ist nur wenig verstanden.

Das molekulare Chaperon Hsp90 essenziell für die Funktion des GR – sowohl stark reduzierte Proteinlevel von Hsp90 als auch dessen Überexpression inhibieren die Aktivität des GR in experimentellen Ansätzen. Somit wurde die Expression der induzierbaren Hsp90α–Isoform in Biopsieschnitten kortikotropher Adenome von Patienten mit Morbus Cushing bestimmt. Die ausserordentlich starke Überexpression in adenomatösen Gewebe im Vergleich zu gesunden Hypophysenzellen hat es ermöglicht, die Rolle von Hsp90 in der Funktion kortikotropher Adenome durch spezifische Inhibitoren zu untersuchen.

Die drei strukturell unterschiedlichen Hsp90–Inhibitoren 17–AAG, Novobiocin und Silibinin zeigten wachstumshemmende Wirkung in AtT–20 Zellen. Diese Effekte gingen mit der Degradation der onkogenen Klientenkinase Cdc2 einher, ein charakteristisches Merkmal für die pharmakologische Inhibition von Hsp90. In Übereinstimmung mit der Literatur führte der auf Geldanamyin basierende, N–terminale Hsp90–Inhibitor 17–AAG zur Degradation des Klientenproteins GR. Überraschenderweise wurde das Proteinlevel des GR weder durch Silibinin noch durch den C–terminalen Hsp90–Inhibitor Novobiocin beeinträchtigt. Dieser Effekt liess vermuten, dass beide Inhibitoren an die selbe Domäne in Hsp90 binden. Es konnte gezeigt werden, dass Novobiocin an Hsp90 gebundenes Silibinin verdrängt, und dass die niedermolekularen Verbindungen die Freilassung des GR auf biochemischer Ebene bedingen. Das führte zu erhöhter Verfügbarkeit von reifem GR in der Zelle, der Glukokortikoid mit hoher Affinität binden konnte. Dieser neuartige, molekulare Mechanismus resultierte in der Potenzierung der transkriptionellen Aktivität des GR. Durch die verstärkte GR–Aktivität wurde die Supprimierung von Adrenokortikotropin sowohl in AtT–20 Zellen als auch in Primärkulturen humaner, kortikotropher Adenome verstärkt. Im Gegensatz hierzu

hatte Silibinin keine Wirkung auf normale Ratten–Hypophysenzellen in Kultur. Letztlich zeigte Silibinin antitumorigene Effekte in einem Maus–Allograftmodell für Morbus Cushing. Auch konnte in diesem Modell eine partielle Unterdrückung der hormonellen Veränderungen erzielt werden, was mit der Reduktion krankheitsbedingter Symptome einherging.

Diese Ergebnisse deuten auf einen pathogentischen Mechanismus des stark überexprimierten Hsp90 hin, der pharmakologisch reversibel ist. Somit könnte der klinisch sichere Hsp90–Inhibitor Silibinin in Zukunft eine Behandlungsmethode von Patienten mit Morbus Cushing darstellen.

3 AIM OF THE STUDY

The molecular chaperone Hsp90 is a validated target in cancer therapy. Furthermore, Hsp90 directly regulates the function of GR. Cushing's disease is caused by corticotroph adenomas in which the GR displays a partial resistance towards glucocorticoids. To date, the pathogenic mechanism that underlies the glucocorticoid resistance in this malignancy is scarcely understood.

The aim of the present work was to investigate the hypothesis that abnormal expression or activity of Hsp90 may cause the reduced hormone responsiveness in corticotroph adenomas. The initial finding that Hsp90 is strongly overexpressed in this malignancy compared to normal human pituitary tissue opened the possibility to investigate its role using distinct classes of specific inhibitors. In the AtT–20 cellular model, the effects of the Hsp90 inhibitors on proliferation and hormone production were characterized. The most promising Hsp90 inhibitor was used to study its effects in primary cultures of human corticotroph adenomas, and its possible influence on normal pituitary cell function *in vitro*. Finally, the mouse allograft model for Cushing's disease was chosen to attempt a proof of principle for the pharmacologic treatment of patients with this severe neuroendocrine condition.

4 INTRODUCTION

4.1 THE ADENOHYPOHYSIS AND CORTICOTROPH ADENOMAS

THE ANTERIOR LOBE OF THE PITUITARY

The adenohypophysis is a major organ of the endocrine system and regulates physiological processes such as stress, growth, reproduction, and lactation. The gland has a diameter of approximately 1 cm in adults and is located within a bony depression of the sphenoid bone called the sella turcica, right below the optic chiasma. During development, upon differentiation and proliferation cells of the anterior wall of the Rathke's pouch give rise to the adenohypophysis. Specialized cells of this organ, when fully developed, produce six hormones: Growth hormone (GH), prolactin, (PRL), follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), and adrenocorticotropin (ACTH) to be released into the bloodstream where they fulfil their role as stimulators of target tissues (Asa and Ezzat, 1998). Corticotrophs is the particular cell type that secretes ACTH and constitutes the central switchpoint of the stress hormone axis. In healthy humans, both stimulatory and repressive mechanisms acting on this cell type facilitate the stress response as well as the reconstitution of homeostasis through intricate regulation of ACTH production and release.

THE STRESS HORMONE AXIS

The hypothalamic–pituitary–adrenal axis (HPA axis) is an integrated multilevel system, responsive to environmental as well as endogenous events, with glucocorticoids (Gcs) being the executive factor and the direct indicator of its activity. The HPA axis enables the organism to adapt to changing demands via the adjustment of metabolic activity on a global scale in order to deal with these events. After the brain has processed stressful situations, such as encounters with predators or an applicant having a job interview, the stress axis will be stimulated – an increase in energy expenditure is expected in order to deal with situations perceived as threatening. Increased energy, however, is provided on the expense of other physiological processes, and homeostasis has to be restored as soon as possible. The functional HPA axis is composed of hypothalamic secretory cells for the synthesis and secretion of corticotrophin releasing hormone (CRH); a hypothalamic pituitary portal system – which is not protected by the blood brain barrier – for the transport of CRH; corticotroph cells of the adenohypophysis expressing the CRH receptor and which are able to process

proopiomelanocortin (POMC) into ACTH for release into the bloodstream; and an ACTH–responsive adrenal cortex to induce the synthesis and release of Gcs (Tsigos and Chrousos, 2002). Gcs have pleiotropic and cell type specific effects, elicited by their molecular target, the ubiquitously expressed glucocorticoid receptor (GR). They influence metabolic activities, the immune system, development, and arousal (Kadmiel and Cidlowski, 2013). It is a remarkable, yet an omnipresent, scheme in biology that a certain effector of stimulating nature simultaneously downregulates its own activity through direct interference with central switchpoints of the pathway it activates: Gcs elicit negative feedback loops on the HPA axis, both centrally and at the pituitary level in corticotroph cells, to prevent overshooting of the stress response as well as to restore homeostasis (**Fig. I – Stress response**).

Corticotroph cells express POMC under the control of the lineage specific transcription factor Tpit (Pulichino et al., 2003). Tpit acts in concert with Pitx to ensure constitutive expression of POMC (Lamolet et al., 2001). In this cell type, POMC is enzymatically cleaved mainly into ACTH to be secreted into the bloodstream (Bertagna, 1994). Under stress, hypothalamic CRH (and also arginine–vasopressin) leads to enhanced production of ACTH through activation of their respective receptor on corticotroph cells – the CRH receptor (and the arginine–vasopressin receptor) (Tsigos and Chrousos, 2002; Vale et al., 1981). The CRH receptor is a G–protein coupled receptor which is linked to the $G\alpha_s$–subunit of G–proteins. The binding of CRH elicits an intracellular signalling cascade via the adenylate cyclase and subsequently protein kinase A (PKA) (Hemley et al., 2007). PKA then activates the transcription factor Nur77 to enhance POMC production (Kovalovsky et al., 2002; Maira et al., 2003; Philips et al., 1997a). The central mechanism for the restoration of homeostasis after stress has waned off is the negative feedback loop of GR at two levels: First, in the hypothalamus, CRH is suppressed to reduce the stimulation of hypohyseal ACTH production (McEwen, 2007). Second and most importantly, GR suppresses POMC gene activity in corticotroph cells, a mechanism called *trans*–repression, which involves the masking of Nur77 to compact the chromatin structure of the POMC gene via recruitment of corepressors (Philips et al., 1997b; Bilodeau et al., 2006).

Controlling the systemic level of ACTH is essential, since it stimulates the adrenal glands to produce Gcs. When the intricate regulatory mechanisms of the stress hormone axis do not function, life threatening hypercortisolism can arise – a condition commonly designated as Cushing's syndrome. Cushing's syndrome can be caused by adenocarcinomas of the adrenal glands which excessively produce Gcs independent of stimulation through circulating ACTH. Pathologically elevated levels of ACTH can also be caused by ectopic ACTH producing

tumors (such as subtypes of lung cancer) (Tritos et al., 2011). However, Cushing's disease is the most common cause of Cushing's syndrome, and it is exclusively caused by corticotroph adenomas (Newell-Price et al., 1998).

Adenomas Of The Anterior Pituitary

Pituitary adenomas constitute up to 15% of intracranial neoplasms, and include those that secrete ACTH (Cushing's disease), GH (acromegaly), PRL (prolactinoma), TSH (hyperthyroidism), and gonadotroph tumors which are mostly non–secreting adenomas. Although growth patterns are not consistently predictable, these monoclonal expansions exhibit a unique and invariably benign proliferative advantage. Tissue invasion and metastases rarely occur. They are associated with severe clinical symptoms due to excessive hormone production or the ability of non–secreting tumors to smother normal pituitary function, which results in a form of hypopituitarism (i.e., lack of pituitary hormones) (Melmed, 2011). The present work focuses on corticotroph adenomas, which excessively secrete ACTH.

Corticotroph Adenomas Cause Cushing's Disease

With an incidence of 80 patients per year and an overall prevalence of approximately 1:100.000 in Germany, Cushing's disease is a rare but severe neuroendocrine condition in which the patients suffer from chronic hypercortisolism caused by corticotroph adenomas. The chronic hypercortisolism causes numerous symptoms such as hypertension, diabetes mellitus, obesity, osteoporosis, myopathy, psychiatric and immune disturbances, and the disease displays with high morbidity and mortality if left untreated (Newell-Price et al., 2006). Treatment of choice is transphenoidal surgery, and if conducted by an experienced surgeon, between 70 to 90% of the patients do not relapse over a period of ten years (Tritos et al., 2011). If the adenoma cannot be located due to its small size (microadenoma), in case of relapse, or if the patient cannot be indicated for surgery due to health problems caused by the chronic hypercortisolism, available treatment options are suboptimal. Radiation therapy presents with a latency period of approximately two years until beneficial effects occur. During that time, the patient requires anti–glucocorticoids (mifepristone) or steroid synthesis inhibitors (ketoconazole) to counter the hypercortisolism. Irradiation may result in the development of hypopituitarism due to collateral damage inflicted on the surrounding healthy pituitary tissue (Vance, 2009). An extreme measure for patients whose life is imminently threatened by the excessive ACTH secretion of the adenoma would be bilateral

adrenalectomy, followed by lifelong Gc–supplementation. However, in up to 50% of the cases, bilateral adrenalectomy results in the development of a Nelson tumor, a very aggressive type of corticotroph pituitary adenomas (Barber et al., 2010). Lastly, and frequently administered since its approval by the Federal Drug Agency in 2013, the somatostatin analog pasireotide is available. In approximately 25% of the patients, it leads to normalization of blood ACTH through direct action on somatostatin receptors expressed on corticotroph adenomas. However, severe side effects are observed in the patients which have to be combated with additional medication (Colao et al., 2012). Altogether, a safe and efficacious medication which targets the pituitary adenoma is needed.

The central pathogenetic mechanism of corticotroph adenomas is their partial Gc–resistance: Only high doses of the synthetic GR–agonist Dexamethasone (Dex) result in the suppression of systemic ACTH in the patients, while the strongly elevated circulating Gc–levels do not (Arnaldi et al., 2003). Thus, GR is expressed in the adenomas, but it can only signal in the presence of very high concentrations of agonist. Mutations of NR3C1, the gene encoding GR, have only sporadically been found in the Gc–resistant adenomas, and thus cannot explain the reduced sensitivity of GR (Lamberts, 2002). To date, a comprehensive understanding of the molecular mechanism that causes the partial Gc–resistance is lacking.

As a basis for the work presented here, it was hypothesized that Hsp90, a direct regulator of GR function, might be involved in the partial Gc–resistance of corticotroph adenomas (**Fig. I – Cushing's disease**).

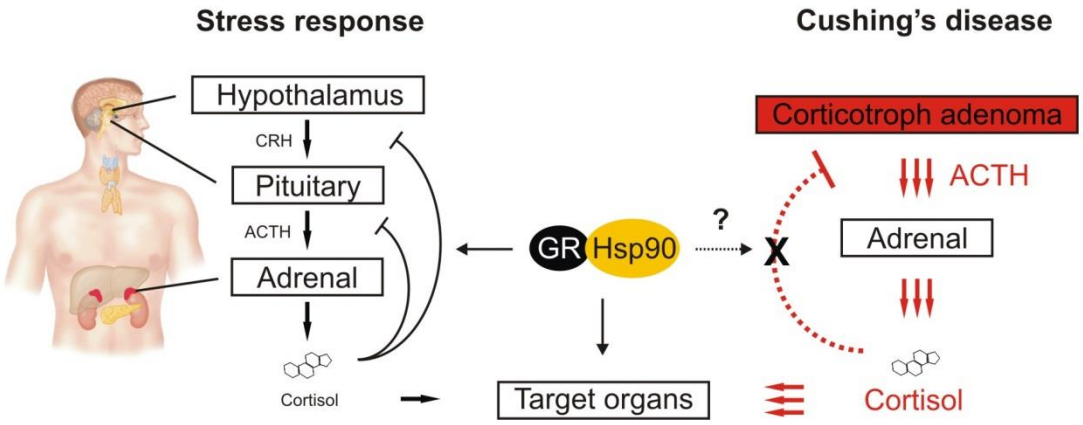

Figure I – Role of GR in the negative feedback loop of ACTH production in health and in patients with Cushing's disease. Hypothalamic and corticotroph GR senses bloodborne Gc–levels for the suppression of HPA axis activity to prevent overshooting of the stress response and to restore homeostasis after stress withdrawal. In Cushing's disease, partially Gc–resistant corticotroph adenomas fail to suppress ACTH. The patients suffer from life threatening hypercortisolism which acts on the sensitive organs. Detrimental mutations of GR only explain sporadic cases of the Gc–resistance, and the molecular mechanism underlying the hormone resistance is scarcely understood. The molecular chaperone Hsp90 ultimately regulates GR function through direct interaction with the receptor at multiple stages during hormone transmission. It was hypothesized here that abnormal Hsp90 function causes the greatly reduced hormone responsiveness in corticotroph adenomas.

4.2 HSP90 IN HEALTH AND DISEASE

CHAPERONES IN PROTEIN FOLDING

Since the onset of molecular biology, the question of how proteins adopt their functional conformation in a living cell has puzzled researchers. It is clear that the laws of thermodynamics fold a protein into a conformation according to its amino acid sequence. But the crowded environment of a cell promotes intermolecular interactions, thus introducing random forces to folding events (Ellis, 2007). This would result in uncontrollable outcomes, and the cell as a highly organized system cannot exist under such conditions. Hence, factors that ensure guided folding are necessary.

Proteins are optimized through evolution to be only marginally stable at the respective growth temperature of an organism. Even for forms of life that thrive at extreme temperatures, a small increase in temperature can cause protein unfolding, entanglement, and unspecific aggregation: Proteostasis is disturbed and puts a severe threat to survival. Thus, the deleterious aggregation of unfolded proteins triggers counter measures: The expression of members of the heat shock family such as chaperones and protein remodelling factors called chaperonins (Richter et al., 2010). These specialized proteins facilitate the folding of newly synthesized proteins and the refolding of misfolded proteins into their active conformation,

aid in assembly and disassembly of macromolecular complexes, target terminally misfolded proteins to degradation via the proteasome, and interact with transport machineries to guide proteins to their proper locations (Zou et al., 2008; McClellan et al., 2005; Young et al., 2003). Hsp90 (heat shock protein 90) fulfills all aspects of a typical heat shock protein under proteotoxic stress (Hartl and Hayer-Hartl, 2009). In addition, it is essential for the function of numerous so–called client proteins – a specific subset of proteins which constitute central regulators of homeostasis, both under normal and pathologic conditions.

HEAT SHOCK PROTEIN 90

Hsp90 is conserved from bacteria to mammals. In mammals, four paralogs constitute the Hsp90–family: Hsp90α and Hsp90β in the cytoplasm, Grp94 in the endoplasmatic reticulum and TRAP1 in the mitochondrial matrix (Sreedhar et al., 2004). The cytoplasmatic alpha and beta isoforms arouse by gene duplication roughly 500 million years ago (human Hsp90α: HSP90AA1 chromosome 14q32.33; human Hsp90β: HSP90AB1 chromosome 6p12). Very early in eukaryotic evolution, Grp94 originated from a paraloguous gene duplication (human Grp94: HSP90B1 chromosome 12q24.2-q24.3), while TRAP1 (human TNF receptor associated protein 1: TRAP1 chromosome 16p13.3) is the product of a very recent gene rearrangement of Hsp90α (Gupta, 1995; Grammatikakis et al., 2002). Although Hsp90α and Hsp90β have 85% sequence identity, and are ubiquituously expressed, there are functional differences unique to each isoform. First, Hsp90α is considered to be highly inducible, but also the constitutive Hsp90β isoform can be enhanced upon sensing of cellular stress (heat shock response) (Richter et al., 2010). This mechanism mainly involves the upregulation of heat shock proteins as well as anti–apoptotic factors through dissociation – and subsequent activation – of heat shock transcription factors (HSFs) from Hsp90 itself (Zou et al., 1998). Hsp90α regulates muscle cell differentiation in zebrafish, and certain aspects of the cell cycle as well as apoptosis (Nakai and Ishikawa, 2001; Lele et al., 1999; Solier et al., 2012). Mice which are homozyguous for a non–functional Hsp90β mutant die during embryogenesis due to a failure in the induction of a placental labyrinth, and the β–isoform displays highly regulated expression patterns during early embryogenesis (Gruppi et al., 1991; Vanmuylder et al., 2002). Furthermore, the two cytosolic isoforms differ in such that they can more or less efficiently chaperone client proteins in yeast, whereas only Hsp90β renders cells sensitive to the N–terminal Hsp90 inhibitor Radicicol (Millson et al., 2007). In sum, diverging functional roles can be attributed to the cytoplasmatic isoforms, but clear distinctions are difficult to

observe due to their compensatory activities. Considering that the work conducted herein has exclusively dealt with the cytoplasmatic proteins, Grp94 and TRAP1 will not be discussed.

Hsp90 consists of three distinct domains: The 25 kD N–terminal domain (NTD), the 35 kD middle domain (MD) and the 12 kD C–terminal domain (CTD) (Pearl and Prodromou, 2006). The eukaryotic NTD is connected to the MD via a highly charged hinge region, which varies in length and composition between species and which is essential for yeast Hsp90 to function (Louvion et al., 1996). Hsp90 participates in general chaperoning activities to restore proteostasis under stressful conditions, in addition to client protein maturation. Chaperone activity has been described for the Hsp90 NTD and CTD, and they function independently of each other to recognize different subsets of substrate to dissociate their aggregation (Scheibel et al., 1998).

In contrast, client protein folding requires extensive conformational changes of full length Hsp90 and a multitude of highly ordered processes of protein–protein interactions (Taipale et al., 2010). All three domains of Hsp90 have been found to interact with client proteins. Hsp90 dimerizes via its CTDs to subsequently undergo conformational changes – it oscillates between open and closed conformations. The state that Hsp90 adopts is ultimately dictated through the binding of cochaperones, which also direct client binding and ATP hydrolysis via the N–terminal ATPase for the maturation of client proteins. The entire process is called the catalytic cycle (Röhl et al., 2013).

HSP90 CLIENT PROTEINS

In all eukaryotes tested, Hsp90 is essential, abundantly expressed and highly inducible by stress. Most proteins do not require Hsp90 for folding *in vivo*. However, more than 300 Hsp90 client proteins are known. They are members of multiple protein families and do not seem to display a common structural feature characterizing them as Hsp90 client proteins (for a regularly updated list of Hsp90 client proteins, refer to www.picard.ch/downloads). An approach to unravel the determinants of client protein recognition by Hsp90 showed that the weak client protein endothelial growth factor receptor (EGFR = ErbB1) can be converted into a strong client protein by mutation of a short stretch of amino acid residues in a putative Hsp90 interaction site (Xu et al., 2005). The mutated sequence in EGFR was identical to that in wildtype ErbB2, a *bona fide* Hsp90 client protein which strongly depends on chaperone function. The dichotomous expression ‘weak’ client and ‘strong’ client was originally coined following the observation that certain client proteins are rapidly degraded when Hsp90 was pharmacologically inhibited, while others remained comparably stable. In contrast to this

convenient example, however, sequence alignments of all known Hsp90 client proteins revealed that the amino acid sequence cannot be the sole factor to determine the dependence of these proteins on Hsp90. Only a recent work has shed light on the principle of client protein recognition, and classification was extended to fit the observation that client proteins exist which display any degree of dependence on Hsp90 – with respect to its interaction, as well as the cellular fate of the client protein subsequent to dissociation from Hsp90 – between weak and strong (Taipale et al., 2012). While the process of maturation for the kinase class of Hsp90 client proteins requires the cochaperone Cdc37 (cell division cycle 37 homolog) (Pearl, 2005), the process itself is still poorly understood. However, the intrinsic thermal stability of the functional kinase domain dictates the dependence of these proteins on Hsp90, rather than their amino acid sequence: There is a negative correlation between strong client proteins, and parameters such as solubility and the availability of solved crystal structures (Taipale et al., 2012). Thus, client proteins can be characterized by binding pockets, catalytic or allosteric domains which are comprised of structural networks with low intrinsic stability – typically, the interaction of Hsp90 with the metastable client proteins is dynamic and keeps them poised for activation (Taipale et al., 2010). In addition to kinases, E3 ubiquitin ligases constitute a prominent class of Hsp90 client proteins (Taipale et al., 2012), but this interaction is the least researched. Furthermore, nuclear hormone receptors are a peculiar subclass of transcription factors that require Hsp90 for maturation (Pratt and Toft, 1997), of which the GR will be discussed in greater detail later.

The Catalytic Cycle Of Hsp90 And The Role Of Cochaperones

The energy necessary for the catalytic cycle of Hsp90 is provided through binding of ATP to a pocket in the NTD of Hsp90, followed by closing of a lid–structure over the nucleotide. The MD subsequently interacts with the NTD – thereby closing the overall chaperone conformation – and participates in ATP hydrolysis, making Hsp90 a split ATPase (Shiau et al., 2006; Ali et al., 2006). Since ATP hydrolysis takes place only when the protomers are twisted around each other, being in contact both at the C–terminus and the NTD, there seems to exist a high degree of communication between the domains of Hsp90 as well as between the protomers. Cochaperones which bind to Hsp90 throughout the catalytic cycle in a sequential manner either enhance – or are permissive for – this communication, thereby influencing its progression and thus, client protein fate (Mayer, 2010; Röhl et al., 2013).

Over 20 cochaperones of Hsp90 are known to date, and they can be divided into three distinct functional classes: (I) The client recruiter cochaperones Hop (Hsp90–organizing protein),

Cdc37, and Sgt1 (suppressor of G2 allele of Skp1) facilitate the loading of Hsp90 client proteins into the complex. All of these cochaperones favour the open conformation of Hsp90 for client loading, and except for Sgt1, they reduce its ATPase activity (Southworth and Agard, 2011; Roe et al., 2004; Kadota et al., 2010). (II) A cochaperone which remodels Hsp90 conformation is Aha1 (activator of heat shock protein 90 ATPase 1). Binding of one Aha1 to an Hsp90 dimer accelerates the closed and catalytically active state (Retzlaff et al., 2010; Li et al., 2013). The autoinhibition of the phosphatase activity of Pp5 (protein phosphatase 5) is relieved upon binding to Hsp90 to facilitate dephosphorylation of the chaperone, which influences its function (Wandinger et al., 2006; Vaughan et al., 2008). (III) The late–acting cochaperone p23 (Sba1; increased sensitivity to benzoquinone ansamycins 1) significantly reduces Hsp90 ATPase activity by trapping the chaperone in the completely closed conformation, with client protein loaded to facilitate maturation (McLaughlin et al., 2006). Upon binding of CHIP (carboxyl terminus of Hsp70 interacting protein) to Hsp90, Hop and p23 are displaced from the chaperone, and the ubiquitin ligase domain of CHIP directs the client proteins for proteasomal degradation (Connell et al., 2001).

About 75% of the cochaperones contain a TPR–motif (tetratricopeptide–motif) to interact with the MEEVD–motif at the very end of the carboxyterminus of Hsp90, while others interact with distinct domains of Hsp90. Thus, at least some cochaperones share the same binding site within Hsp90. Considering that Hsp90 is 10–1000 times more abundant than any cochaperone, the existence of mixed and asymmetric complexes *in vivo* is likely (Röhl et al., 2013). Depending on the cellular environment, such as cochaperone expression and client protein abundance, certain types of complexes may dominate inside different cell types or under pathologic conditions.

Through attempts to describe the influence of cochaperones on the full catalytic cycle, a picture has emerged through careful assembly of insights gained by the utilisation of a vast array of biochemical and structural biology techniques. All information was gained with purified proteins, and nuclear hormone receptors were the client proteins used in these highly artificial models – other classes of Hsp90 client proteins may require different mechanisms.

Current knowledge suggests that the two TPR–domains in Hop bind to the MEEVD–domain of both Hsp70 – a promiscuous chaperone which is required for specific and folding/unfolding steps of nuclear hormone receptors at multiple levels (Pratt et al., 2006; Kirschke et al., 2014) – and Hsp90 to facilitate client protein loading. The second MEEVD of the Hsp90 dimer then binds a TPR–motif containing PPIase (peptidylprolylisomerase). The

activator Aha1 binds upon release of Hop and Hsp70. Then, p23 displaces Aha1 and the nucleotide is trapped in the closed chaperone conformation to be hydrolyzed. Only after ATP–hydrolysis, the client and cochaperones are released (Scheufler et al., 2000; Retzlaff et al., 2010; Li et al., 2011). Intriguingly, very recently it has been shown that hydrolysis of ATP by Hsp90 was required for the release of GR from Hsp70, and that the chaperoning activity of Hsp90 on GR did not require ATP hydrolysis (Kirschke et al., 2014). In sum, the scheme might not be complete and function in a more complex manner *in vivo*. For example, it was to date not possible to integrate Hsp70/Hop into the catalytic cycle of kinases, since most kinases directly interact with Cdc37 and this interaction is abolished upon inhibition of Hsp90 (Taipale et al., 2012). On the other hand, Hsp70 inhibitors lead to the degradation of Hsp90 client proteins (Taldone et al., 2014; Wang et al., 2010; Powers et al., 2008).

A further layer of control of chaperone cycle progression and function to adapt to differential cellular needs is through post–translational modifications (Mollapour and Neckers, 2012). Modifications within the different domains were shown to affect global aspects of Hsp90 function: Phosphorylation, sumoylation, and S–nitrosylation influenced Hsp90 ATPase activity, cochaperone binding, dimerization and conformational equilibrium, thereby either constituting switch points for the communication between the domains or modulating the function of the modified domains (Soroka et al., 2012; Retzlaff et al., 2009; Martinez–Ruiz et al., 2005; Mollapour et al., 2010; Beebe et al., 2013; Mollapour et al., 2014). As will be introduced later, it was tempting to assume that small molecule inhibitors which bind to the different domains of Hsp90 might thus elicit distinct effects on chaperone function.

HSP90 IN CANCER

Hsp90 is overexpressed in many solid tumors and haematological malignancies (Isaacs et al., 2003; McDowell et al., 2009). It serves to buffer intracellular stress caused by the transformation and oncogenicity, and it promotes the functional state of oncogenic client proteins which are amplified, mutated, or mislocated (Trepel et al., 2010). Hsp90 in cancer cells exists in a hyperactive complex as compared to that found in normal cells, i.e. the ATPase activity of cancer cell Hsp90 is greatly enhanced (Kamal et al., 2003). It is assumed that the composition of cancer cell Hsp90 complexes as well as post–translational modifications of Hsp90 are the cause for the observed hyperactivity. This furthermore implies that malignant cells display increased dependence on Hsp90, which is reflected by the fact

that mutant oncoproteins are particularly unstable and consequently require strongly increased chaperoning activity (Whitesell and Lindquist, 2005).

Many of the known client proteins constitute central hubs of pathways that govern the five hallmarks of cancer (Whitesell and Lindquist, 2005). Malignant cells need to proliferate, and they require unlimited replicative potential to do so. Cancer cells must be able to trigger survival pathways, withstand stress (such as nutrient deprivation or hypoxia), and they need to induce vascularisation for further growth. At the final stage, cancer cells must evade restraining tissue architecture to be able to metastasize (**Fig. II**).

Therefore, pharmacologic inhibition of Hsp90 simultaneoulsy impedes multiple oncogenic pathways, depriving cancer of its hallmark characteristics. Two distinct classes of Hsp90 inhibitors to combat cancer will be introduced in the following paragraphs: N–terminal and C–terminal Hsp90 inhibitors.

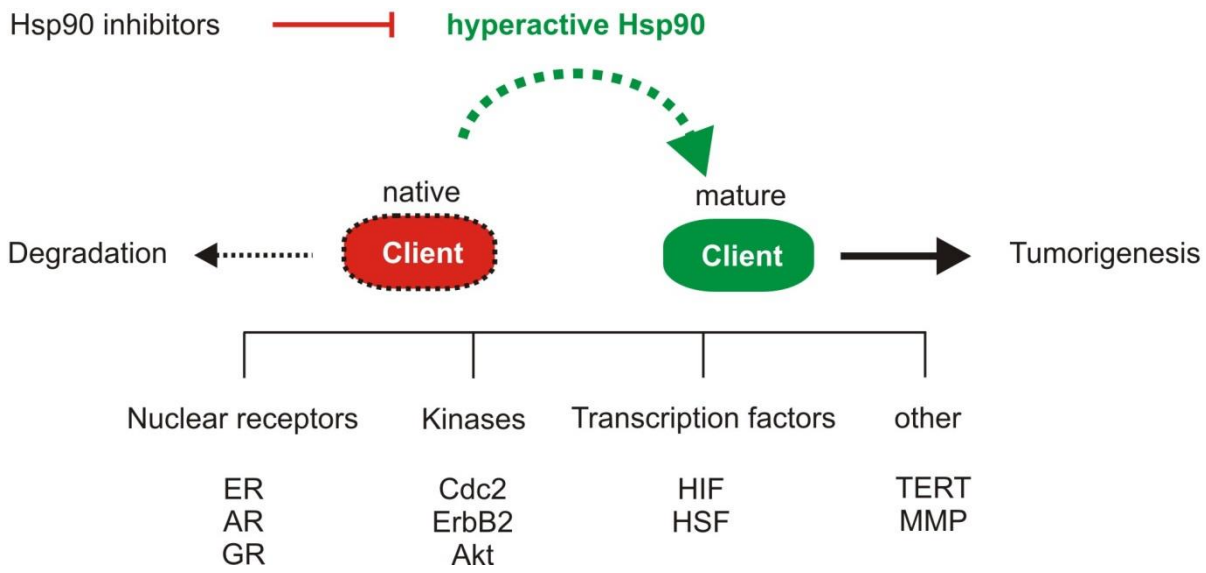

Figure II – The hyperactive Hsp90 complex in cancer cells promotes tumorigenesis through activation of oncogenic client proteins. Hsp90 client proteins encounter the chaperone in their native conformation. The catalytic cycle facilitates conformational changes in the clients which poise them for activation, thus driving tumorigenesis. Prominent oncogenic client proteins include: The steroid hormone receptors estrogen receptor (ER), androgen receptor (AR) and GR (Bagatell et al., 2001; Vanaja et al., 2002; Segnitz and Gehring, 1997); Cell division cycle 2 homolog (Cdc2), human epidermal growth factor receptor (ErbB2) and protein kinase B (Akt) (Garcia-Morales et al., 2007; Mimnaugh et al., 1996; Basso et al., 2002); The heat shock factors (HSF) and hypoxia inducible factors (HIF) (Zou et al., 1998; Minet et al., 1999); Matrix metalloproteinases (MMP) (Eustace and Jay, 2004)) and telomerase (TERT) (Holt et al., 1999). Pharmacologic inhibition of Hsp90 leads to client protein degradation in cancer cells.

N–TERMINAL HSP90 INHIBITORS

The structurally unrelated natural products Geldanamycin (*Streptomyces hygroscopicus*) and Radicicol (*Diheterospora chlamydosporia*) were shown to bind to the N–terminal ATPase

domain of Hsp90, thereby depriving the catalytic cycle of ATP (Trepel et al., 2010). In the presence of N–terminal Hsp90 inhibitors most, if not all, proteins which require Hsp90 for proper folding consequently lose activity, aggregate, and are targeted for ubiquitination and subsequent degradation through the proteasome. It quickly became clear that these molecules are potent anticancer agents, and that their antitumorigenic activity is based on the targeted degradation of numerous oncogenic client proteins (Xu and Neckers, 2007).

The nucleotide binding site in the N–terminus of Hsp90 is special because ATP is bound in a kinked conformation. The so–called Bergerat ATP binding–fold is unique to a superfamily of ATPases also including DNA gyrase and the mismatch repair protein MutL (GHL–family) (Dutta and Inouye, 2000). With Hsp90 being the only eukaryotic protein of this superfamily (Roe et al., 1999), N–terminal Hsp90 inhibitors bind the chaperone with astonishing affinity and specificity. In addition, the previously mentioned, hyperactive species of Hsp90 detected in cancer cells exhibits strongly increased affinity towards these compounds as compared to the affinity of Hsp90 in normal cells – this class of inhibitors preferentially lead to cancer cell death (Kamal et al., 2003; Moulick et al., 2011; Mollapour et al., 2014). The tumor selectivity achieved with N–terminal Hsp90 inhibitors gained tremendous attention from the pharmaceutical industry: Nine structurally unrelated compounds which target the ATPase domain are currently in clinical development to combat cancer, with positive outcomes (www.clinicaltrials.gov).

C–TERMINAL HSP90 INHIBITORS

The necessity to develop an additional class of Hsp90 inhibitors can be explained by the fact that several N–terminal Hsp90 inhibitors exhibit severe side effects such as liver, intestinal, pulmonary, and ocular toxicity. The clinical development of many of them was discontinued. At the cellular level, N–terminal Hsp90 inhibitors elicit the heat shock response, which includes the upregulation of anti–apoptotic genes such as Hsp70 (Zou et al., 1998; Richter et al., 2010). The induction of Hsp70 promotes the chemoresistance of cancer cells and desensitizes for Hsp90 inhibition itself (Gabai et al., 2005; Chatterjee et al., 2012; Powers et al., 2008; Zaarur et al., 2006). Thus, these drugs trigger a negative feedback loop which induces resistance towards their own mechanism of action: Higher doses are necessary, which in turn are expected to show more severe side effects.

The natural antibiotic Novobiocin (*Streptomyces niveus*) was thought to be an additional inhibitor of the N–terminal ATPase domain of Hsp90, since it inhibits DNA gyrase (Ali et al., 1993). Surprisingly, affinity purification experiments, analyses with trypsin digestion patterns,

and molecular modelling have shown that Novobiocin binds to a cryptic nucleotide binding pocket in the C–terminal domain of Hsp90, and that cancer cells undergo apoptosis due to the degradation of oncogenic client kinases upon inhibition with this small molecule (Marcu et al., 2000a; Marcu et al., 2000b; Matts et al., 2011), without induction of the heat shock response (Donnelly and Blagg, 2008). This functional discrepancy was the first evidence that it is possible to modulate Hsp90 activity in specific ways using compounds that bind to different domains of the chaperone. Additional work revealed that after binding, Novobiocin forces Hsp90 into a conformation that promotes the release of, for example, the client heme–regulated eIF2α kinase before it completes maturation (Yun et al., 2004). The composition of cochaperone–Hsp90 complexes in the presence of C–terminal Hsp90 inhibitors differs greatly from that observed after binding of N–terminal Hsp90 inhibitors (Söti et al., 2002; Allan et al., 2006; Smith et al., 1995; Thulasiraman and Matts, 1996). Furthermore, the recent finding from Kirschke et al. that the activation of GR which was partially unfolded by Hsp70 requires the CTD of Hsp90 independent of ATP–hydrolysis (Kirschke et al., 2014) strongly suggests that C–terminal Hsp90 inhibitors could modify the chaperoning activity in a distinct fashion compared to N–terminal Hsp90 inhibitors: With respect to GR, the hydrolysis of ATP by the N–terminal Hsp90 ATPase is uncoupled from the chaperoning activity mediated by the C–terminal domain. Yet, also for this mechanism, ATP–hydrolysis by Hsp90 is essential for the activation of GR, namely to release it from Hsp70 for subsequent maturation.

Silibinin, the major constituent and active ingredient of extracts of milk thistle (*Silybum marianum*) seeds, was also shown to be an Hsp90 inhibitor, and its effects recalled those elicited by C–terminal Hsp90 inhibitors (Zhao et al., 2011).

THE GLUCOCORTICOID RECEPTOR

GR is a nuclear hormone receptor encoded by the NR3C1 gene. Transcription of this gene is translated into three GR isoforms, of which only the alpha isoform can bind Gcs. Alternative splicing results in a unique 15 amino acid stretch within the carboxy–terminus of GRβ, the presence of which disturbs the allosteric network of the ligand binding pocket of the receptor, thus rendering it unable to bind Gcs. This isoform is expressed in certain tissues, can exert dominant negative effects on GRα in experimental settings, but its role *in vivo* is under debate (Bamberger et al., 1995; Vottero and Chrousos, 1999; Carlstedt–Duke, 1999). Of relevance for this work, GRβ was found to be equally expressed in corticotroph adenomas as compared to normal pituitary (Dahia et al., 1997) and likely does not play a role in the pathogenic mechanism of Cushing's disease. The P–isoform is truncated at the carboxy terminus (Krett et

al., 1995). It has been detected in some types of Gc–resistant tumors and haematological malignancies, but was shown to enhance GRα activity in experimental settings (Parks et al., 1998; Moalli et al., 1993; de Lange et al., 2001). No reports exist on the expression of GR–P in corticotroph adenomas. Since only the isoform capable of binding Gcs was investigated here, the designation GR stands henceforth for GRα.

As a typical nuclear receptor, GR consists of a ligand binding domain (LBD), a DNA binding domain, and activator function domains. In addition to fulfilling the action that gave the domains their name, they can and do interact with other proteins, including other GR monomers for dimerization (Nicolaides et al., 2010). Unliganded GR (apo–GR) is mainly localized in the cytoplasm. Upon binding to its ligand (holo–GR), the receptor rapidly translocates into the nucleus to modulate target gene activity. Two mechanistically different actions are exerted by the activated GR: *Trans*–activation and *trans*–repression (Nicolaides et al., 2010). In *trans*–activation, a homodimer of GR directly binds to GR response elements (GREs) in the promoter region of target genes to interact with members of the nuclear receptor coactivator families such as NCoA–1 (SRC–1), NCoA–2 (TIF2, GRIP1), and the cointegrator p300/CBP. The transcription of target genes is induced and/or enhanced through the subsequent relaxation of the chromatin structure, as well as augmentation of promoter clearance and recruitment of RNA polymerase II (Voegel et al., 1996; Onate et al., 1995; Chakravarti et al., 1996; McKenna and O'Malley, 2002). Important insights into the mechanistic aspects of transcriptional control conferred by GR have been made with constructs containing the mouse mammary tumor virus long terminal repeat (Cordingley et al., 1987), which was also used in the present work to detect GR *trans*–activation.

In *trans*–repression, the GR antagonizes the activating function of a subset of transcription factors including Nur77, NFKB, and AP1 (Adcock et al., 1999; Konig et al., 1992; Philips et al., 1997b; Heck et al., 1994). This mechanism does not involve the binding of dimeric GR to DNA but binding to the activated transcription factors on their respective response elements as a monomer, which induces a distinct conformation of GR to allow for recruitment of histone deacetylases (HDACs) and other corepressors to shut down target gene transcription via chromatin remodelling and/or inhibition of RNA polymerase II promoter clearance (Ito et al., 2000; Bilodeau et al., 2006; Nissen and Yamamoto, 2000; De Bosscher et al., 2003). The anti–inflammatory actions of GR are mediated mainly through *trans*–repression (Hayashi et al., 2004). The negative feedback mechanism of Gcs on the HPA axis at the hypophyseal level (normal corticotroph cells) acts through this mechanism as well (Philips et al., 1997b;

Bilodeau et al., 2006) and was investigated in the work presented here, since corticotroph adenoma cells are resistant to Gc–feedback.

THE ROLE OF HSP90 IN GLUCOCORTICOID RECEPTOR FUNCTION

In order to fulfil its activity as a transcription factor, GR requires multifaceted interaction with Hsp90 both before and after binding to Gcs. As an overview depicted in Figure III, Hsp90 facilitates receptor maturation into its high–affinity binding state for steroid, mediates the rapid translocation of activated GR into the nucleus, and impacts transcriptional complexes on the target genes which contain GR (**Fig. III**).

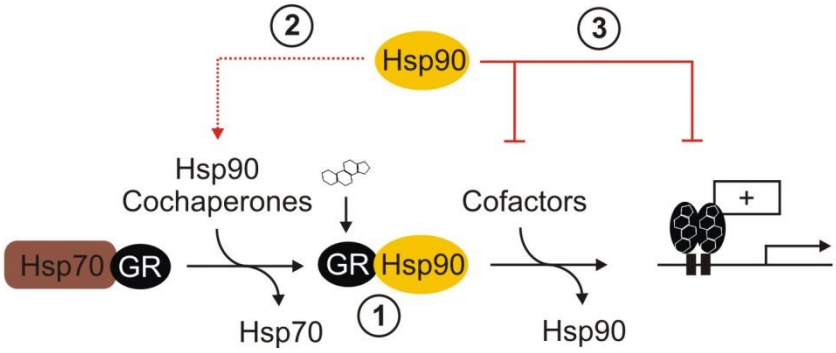

Figure III – Rationale of Hsp90 in GR function. (1) Physiological levels of Hsp90 are essential for the maturation of the GR–LBD into its high affinity steroid binding conformation. Through continuous interaction, the chaperone sequesters GR in the cytoplasm until the binding of agonist triggers a cascade of events, including the Hsp90–dependent transport into the nucleus, where the chaperone is replaced by transcriptional cofactors. In experimental settings, both reduced (2) and strongly elevated expression levels (3) of Hsp90 impede GR signalling at different steps.

The quest for the molecular target of Gcs started at the biochemical level. A heteromeric protein complex that bound this steroid was identified using sucrose gradient centrifugation of cellular lysate (Baxter and Tomkins, 1971). The complex was dynamic, and it consisted of three proteins of which only one was found to actually bind steroid (Yamamoto et al., 1974; Housley et al., 1985). The other two proteins were a dimer of Hsp90 (Joab et al., 1984). An inhibitory role in GR signalling was initially attributed to Hsp90 due to the observations that cellular lysate containing GR bound to Hsp90 could not bind to DNA. Following artificial dissociation from Hsp90 in cellular extracts through elevated temperature, ionic strength, pH, or dilution, GR did bind to DNA (Harmon et al., 1988; Nielsen et al., 1977). However, GR which was dissociated from Hsp90 under such conditions did not bind Gcs in the first place (Bresnick et al., 1989). Since Gcs regulate a plethora of processes, the ability of GR to sense physiological Gc–levels in order to shape the cellular responses was considered the logic basis for its function. Furthermore, Gc–binding is necessary for GR to bind to specific response

elements in DNA in cells (Becker et al., 1986). Later, a system in which the interaction between Hsp90 and GR inside a living cell could be investigated more in detail was wanted. A yeast strain was created, in which the protein level of Hsp90 was reduced to 5% of that of the WT strain (Picard et al., 1990). While yeast cells do not have endogenous steroid hormone receptors, mammalian steroid hormone receptors are functional when overexpressed (Schena and Yamamoto, 1988). The strongly reduced expression of Hsp90 rendered GR free from Hsp90 – but merely being dissociated from Hsp90 in the absence of hormone did not confer activation on GR function in the cellular setting. While GR was not targeted for degradation in the mutant strain, only more than 20–fold higher concentrations of agonist activated GR in the mutant compared to GR expressed in the WT strain (Picard et al., 1990). In a different approach, a temperature sensitive Hsp90 mutant displayed WT activity at ambient growth temperature in yeast with respect to chaperoning activity of GR. At elevated temperature however, this mutant lost chaperoning activity, and hence GR did not function (Nathan and Lindquist, 1995). It was suggested that GR requires continuous interaction with Hsp90 to maintain its high–affinity conformation to bind Gcs, and that binding of Gcs to GR must constitute a dominant conformational switch that acts on the entire receptor.

In the following years, different experimental approaches succeeded in showing that ATP together with purified Hsp40, Hsp70, Hop and Hsp90 could reassemble a complex to refold salt–stripped (native) GR into its high–affinity binding state (Dittmar and Pratt, 1997). GR thus reconstituted quickly lost its high–affinity conformation at elevated temperature, and p23 was shown to stabilize the high–affinity state through the creation of a stable apo–GR::Hsp90 complex, which kept GR in a conformation able to bind agonist (Dittmar et al., 1997). Rabbit reticulocyte lysate (RRL), which abundantly contains the Hsp90 cochaperone complex, could also mature GR when an ATP–regenerating system was included (Dittmar et al., 1996). RRL contains cochaperones in addition to the purified protein assembly system (Hsp40 – Hsp70 – Hop – Hsp90 – p23). Proteins such as Aha1, immunophilins, and protein phosphatases greatly influence client protein maturation, and this system is more reminiscent of intracellular folding processes than the purified protein assembly system (Pratt et al., 2006).

Common knowledge dictated that binding of Gcs to GR causes dissociation of the receptor from Hsp90 (Pratt and Toft, 1997). Due to technical limitations, it was not possible to detect the dynamic interactions between holo–GR and Hsp90 after the agonist elicited the conformational change in the receptor which allowed for translocation and cofactor recruitment. An elegant work by Czar *et al.* demonstrated that Hsp90 is required for the rapid translocation of holo–GR into the nucleus in order to act as a transcription factor (Czar et al.,

1997). It has been shown that the N–terminal Hsp90–inhibitor Geldanamycin quickly abolished the agonist binding activity of GR, which was followed by proteasomal degradation of the receptor after prolonged exposure to the inhibitor (Whitesell and Cook, 1996; Segnitz and Gehring, 1997). To circumvent the influence of Geldanamycin on GR prior to agonist binding, cells were incubated with Dexamethasone at low temperature to allow for binding to GR. Subsequently, Geldanamycin was added before the cells were exposed to growth temperature. The receptor did not translocate to the nucleus in an active process, only by slow, passive diffusion (Czar et al., 1997). It was thus shown that GR continues to require Hsp90 for certain functions also after the structural changes induced by the binding of agonist. It was later shown that holo–GR indeed interacts with Hsp90 (Lorenz et al., 2014; Fang et al., 2006; Kirschke et al., 2014).

Long after it was known that Hsp90 interacts with the LBD of GR (Gehring and Arndt, 1985; Xu et al., 1998; Howard et al., 1990), Fang *et al.* provided insights into how the dynamic interactions of holo–GR with Hsp90 occur (Fang et al., 2006). The LBD of GR consists of an allosteric network that transmits hormone–induced conformational changes inside the ligand binding pocket to a hydrophobic groove on its surface. This groove interacts with conserved LxxLL–motifs in amphipathic α–helices of coregulators, Hsp90, and GR itself when bound to agonists (Bledsoe et al., 2002; Kauppi et al., 2003; Fang et al., 2006). It was demonstrated that apo–GR interacts with the helix motif 10 of Hsp90 (Hsp90–HM10), which forces the flexible helix 12 of GR (GR–H12) to occupy the hydrophobic groove to stabilize it. Since communication between the ligand binding pocket and the hydrophobic groove is bidirectional, a stable groove likewise stabilizes the ligand binding pocket, thus preventing it from collapsing – the high affinity conformation of GR towards its agonist is preserved. Upon binding of agonist, the flexible GR–H12 displaces Hsp90–HM10 and allows the hydrophobic groove of holo–GR to interact with Hsp90 via its helix motif 9 (Hsp90–HM9), again to protect against collapse (Fang et al., 2006).

To further understand the role that Hsp90 plays in modulating the highly dynamic allosteric system of the GR–LBD, a comprehensive screen for GR mutants which increase hormone responsiveness was conducted in yeast (Ricketson et al., 2007). Single point mutations that increased GR activity were identified, and these mutations were located inside the above mentioned allosteric network. In contrast to the increased transcriptional activity observed in yeast however, these mutants almost exclusively displayed impaired GR signalling in mammalian cells. This finding likely reflects the flexible effects of GR in high eukaryotes such as *trans*–activation, *trans*–repression, and mixed agonist/antagonist activities of RU–

38486, whereas this ligand is a pure antagonist in yeast. In any case, the point mutations conferred stable structural changes in the GR–LBD independent of Hsp90: When the mutants were expressed in bacteria, which lack the chaperone machinery to mature nuclear receptors (with the exception of the Hsp90 homolog HtpG), they displayed increased agonist binding capacity as compared to WT–GR (Ricketson et al., 2007). It can be argued that the stable changes induced by the mutations on the allosteric network annihilate the structural flexibility conferred on WT–GR through Hsp90 and cofactors to function in a cell type specific manner. These findings demonstrated multifacetted roles of Hsp90 in the function of GR.

Artificial overexpression of Hsp90 has a negative impact on GR signalling in experimental settings (Kang et al., 1999; Freeman and Yamamoto, 2002). In contrast to low levels of Hsp90, where the partially denatured GR bound to Hsp70 cannot be matured into its high–affinity binding conformation, the overexpression of Hsp90 interferes with later steps in GR activity. High nuclear Hsp90 levels impeded hormone responsiveness through the dissociation of holo–GR from its cognate response elements (Kang et al., 1999). Furthermore, if Hsp90 was artificially targeted to the vicinity of a GRE–containing promoter, it displaced transcriptional cofactors from GR in transcriptional complexes, likewise leading to a reduction in GR activity (Freeman and Yamamoto, 2002). Since endogenous Hsp90 is recruited to GR–responsive genes (Freeman and Yamamoto, 2002), it is possible that the abnormally high expression levels of Hsp90 observed in cancer might impact holo–GR.
In the work presented here, evidence is provided that strongly increased levels of Hsp90α in corticotroph adenoma cells negatively interfere with GR signalling in a step prior to the binding of hormone, and that C–terminal Hsp90 inhibitors reverse this inhibitory mechanism.

5 MATERIALS & METHODS

5.1 REAGENTS

Chemicals and Reagents	Company
17–AAG	Tocris
^{3}H–Dex	Perkin Elmer
ABC (Avidin Biotin Complex)	Vector
Acetic Acid	Sigma
Acridine orange	Sigma
Acrylamide/Bis–acrylamide 30% solution	Sigma
ACTH	Bachem
AG1–X8	BioRad
Agar	Life Technologies
Agarose	Roth
Ampicillin	Sigma
APS	Sigma
AtT–20 clone D16v–F2	ATCC
B–Mercaptoethanol	Merck
Bradford Protein Assay	BioRad
BSA	Invitrogen
Charcoal	Roth
Chloramin T	Merck
Clarity ECL	BioRad
DAB	Sigma
DAPI	Sigma
Developer Solution	Kodak
Dextran T70	Roth
DMEM	Gibco
DMSO	Roth
DTT	Sigma
Dynabeads Protein G	Invitrogen
EDTA	Sigma
Entellan	Merck
Ethidium Bromide	Sigma
EtOH 100%	Roth
FCS	Gibco
Fixer Solution	Kodak

Chemicals and Reagents	Company
Forskolin	Sigma
Glucose	Merck
Glycerol	Sigma
HCl	Roth
HEPES	Sigma
Hydrogen Peroxide Solution 30%	Sigma
^{125}I	PerkinElmer
Igepal CA-630	Sigma
IPTG	Sigma
Isopropanol	Sigma
L–Glutamine	Biochrom
Lipofectamine2000	Invitrogen
Luciferin	PJK
MEM vitamins	Biochrom
Milk powder	Roth
Na_2MoO_4	Sigma
NaCl	Roth
NaOH	Roth
Novobiocin	Calbiochem
ONPG	Sigma
Optimem	Gibco
Partricin	Biochrom
Passive Lysis Buffer	Promega
PEG 6000	Merck
Peptone	MP Biomedicals
Penicillin + Streptomycin Mix	Biochrom
PFA	Sigma
Plasma ACTH Kit ImmuChem	MP Biomedicals
Plasma Corticosterone Kit	DRG Instruments
Plasmid Extraction Kit	Qiagen
Poly–L–Lysine	Sigma
Poly–L–Lysine Coated Slides	Sigma
Polystyrene Microtiter Plates	Corning
ProLong Antifade Gold	Invitrogen
Propidium Iodide	Sigma
Protease Inhibitor Cocktail	Sigma
Protein G Dynabeads	Invitrogen

Chemicals and Reagents	Company
PVDF Membrane	Millipore
QuikChange II XL Mutagenesis Kit	Agilent Technologies
RNAse A	Promega
RU–38486	Tocris
Sample Buffer (4x)	Roth
Scintillation Vials	Roth
SDS	Roth
Silibinin	Sigma
Silicur	Hexal
Sodium Bicarbonate	Sigma
Sodium Carbonate	Sigma
Sodium Disulfite	Merck
Sodium Hydroxide	Sigma
Sodium Molybdate	Sigma
Substrate Reagent for HRP	R&D
Sulfuric Acid	Sigma
TEMED	Sigma
Toluidine Blue	Sigma
Tris Base	Roth
Triton X–100	Roth
Trypsin/EDTA	Biochrom
Tween–20	Sigma
Ultima Gold Scintillation Fluid	Perkin Elmer
WST–1 Assay	Roche
Xylol	Roth
Yeast extract	MP Biochemicals

5.2 SOLUTIONS

Buffer	Composition
BCB	Na_2CO_3 : 3.03 g l^{-1} $NaHCO_3$ 6 g l^{-1} adjust to pH 9.5
Charcoal/Dextran Suspension	Charcoal : 100 g l^{-1} Dextran T70 : 10 g l^{-1} in 10 mM Tris buffer pH 7.8
HDB buffer	Glucose : 10 mM NaCl : 137 mM KCl : 5 mM Na_2HPO_4 : 0.7 mM HEPES : 25 mM adjust to pH 7.3 Partricin : 500 µg l–1 Penicillin/Streptomycin : 10^5 U l^{-1}
LB medium	Peptone : 10 g l^{-1} Yeast extract : 5 g l^{-1} NaCl : 5 g l^{-1} NaOH 1M : 2 ml l^{-1} adjust to pH 7.0
Lower Tris Buffer	Tris : 182 g l^{-1} SDS : 4 g l^{-1} adjust to pH 8.8
ONPG buffer 2x	Na_2HPO_4 1M : 55.3 ml NaH_2PO_4 1M : 29.3 ml DDW : 339.2 ml $MgCl_2$ * $6H_2O$: 154.5 mg ONPG : 500.0 mg b-Mercaptoethanol 14M : 2.5 ml freeze aliquots at -20 °C
PBS	NaCl : 8 g l^{-1} KCl : 0.2 g l^{-1} $Na_2HPO_4.2H_2O$: 1.44 g l^{-1} KH_2PO_4 : 0.2 g l^{-1} adjust to pH 7.4
PEG buffer	Polyethylenglycol 6000 : 60 g l^{-1} in phosphate buffer
PFA 4% in PBS	Paraformaldehyde : 40 g l^{-1} in PBS or TBS freeze aliquots at –20 °C

Buffer	Composition
Phosphate Buffer	Na_2HPO_4 * 2 H_2O : 7.06 g l^{-1} NaH_2PO_4 * 2 H_2O : 1.32 g l^{-1} adjust pH to 7.3
RIPA cell lysis buffer	Tris HCl pH 8 : 50 mM NaCl : 150 mM NP–40 :1% Sodium Deoxycholate : 0.5% SDS : 0.1%
Running Buffer	Tris : 3.03 g l^{-1} Glycine : 14.42 g l^{-1} SDS : 1.00 g l^{-1} adjust to pH 8.3
Running Gel 10% (SDS-PAGE)	DDW : 6.6 ml Acrylamide : 8 ml Lower Tris-Base pH 8.8 :5 ml Ammonium persulfat 10% : 0.2 ml Temed : 0.008 ml
Stacking Gel 4% (SDS-PAGE)	DDW : 4.1 ml Acrylamide :1 ml Upper Tris Buffer pH 6.8 : 0.75 ml Ammonium Persulfat 10% : 0.06 ml Temed : 0.006 ml
TEDGM buffer	Tris : 10 mM NaCl : 50 mM EDTA : 4 mM Na_2MoO_4 : 10 mM glycerol : 10% DTT : 1 mM, add freshly prior to use
Towbin Transfer Buffer	Tris : 3.03g l^{-1} Gycine : 14.42 g l^{-1} Methanol : 150 ml l^{-1}
TBS	Tris : 2.42 g l^{-1} NaCl : 8 g l^{-1} adjust to pH 7.6
TBST	Tris : 2.42 g l^{-1} NaCl : 8 g l^{-1} Tween 20 : 1 ml l^{-1}
Upper Tris Buffer	Tris : 60.5 g l^{-1} SDS : 4.0 g l^{-1} adjust to pH 6.6

5.3 ANTIBODIES

Target	Name	Company	Application	Conjugation
GR	H–300	Santa Cruz	IP, ICC	-
GR	M–20	Santa Cruz	IB	-
Hsp90α	EPR3953	Epitomics	IHC	-
Hsp90α/β	H90–10	Thermo Scientific	IB, ICC	-
Hsp90α	ADI–SPA 771	Thermo Scientific	IB, IP	-
Hsp90α/β	clone 68/Hsp90	BD Biosciences	ELISA	-
Cdk1	clone 1/Cdk1/Cdc2	BD Biosciences	IB	-
β–actin	8H10D10	Cell Signaling	IB	-
ACTH		MPI of Psychiatry	RIA	-
non–immune	Sc–2027	Santa Cruz	IP	-
mouse–IgG	7076S	Cell Signaling	IB	HRP
rabbit–IgG	7074S	Cell Signaling	IB	HRP
rabbit–IgG	BA1000	Vector	IHC	biotinylated
rabbit–IgG	A–11012	Life Technologies	ICC	AlexaFluor 594
mouse–IgG	A–11001	Life Technologies	ICC	AlexaFluor 488
rabbit–IgG	AP132	Millipore	RIA	-

5.4 METHODOLOGY

IMMUNOHISTOCHEMISTRY

In order to investigate the expression and subcellular localization of Hsp90α in human corticotroph adenomas as compared to normal human pituitary tissue, immunohistochemistry was conducted. 8 µm sections of frozen normal and adenoma tissue were cut in a cryostat (Leica CM3050 S) and bedded on poly–L–lysine coated glass slides. The tissue was fixed in freshly prepared cold PBS with 4% PFA, dehydrated and stored at 4°C until use. For the experiments, the sections were incubated for 5 min in TBS, followed by 30 min blocking in TBS with 10% goat serum. Endogenous peroxidase activity was blocked by 15 min incubation with TBS and 1% H_2O_2, and sections were incubated with antibody to Hsp90α (EPR3953 1:200 – Epitomics) diluted in TBS and 3% BSA ON at 4°C. After 3 washes in TBS, the biotinylated secondary antibody (BA1000 1:500 – Vector) was diluted in blocking solution and incubated for 30 min at RT. After 3 washes in TBS, the slides were incubated for 30 min with the ABC complex. The ABC complex binds to the biotinylated secondary antibody, and since there are multiple biotin motifs on each antibody, the ABC complex which contains HRP enhances the signal in comparison to regular HRP–conjugated antibodies. The ABC complex was prepared 30 min prior to use in Tris–buffer to allow complex formation. After 3 washes in TBS, the slides were immersed in freshly prepared DAB (1 mg ml^{-1} in DDW) supplemented with 0.01% H_2O_2 until the signal was visible by eye. For each section stained for Hsp90α, the adjacent section was incubated in parallel without primary antibody to control for background. Only those sections which did not give a signal for the control were considered for analysis. After thorough washes in TBS, the slides were counterstained with toluidine blue (1 mg ml^{-1} in DDW), which stains the nuclei pale blue to allow for sighting of tissue organization. Excess color was removed by immersing the cells in 70% ethanol supplemented with acetic acid, followed by dehydration and fixation in xylol. Slides were coverslipped in Entellan (VWR), and evaluated using the Zeiss Axioskop II.

CELL CULTURE

The murine corticotroph tumor cell line AtT–20 is a well characterized bona fide model for corticotroph function. AtT–20 clone D16v–F2 cells were obtained from ATCC and grown for maintenance in what will be referred to as cell culture medium: DMEM (Gibco 41965) supplemented with 10% FCS (Gibco – 10270 heat–inactivated for 1 hour at 55°C), 2 mM L–glutamine (Biochrom), 100 U ml^{-1} penicillin/streptomycin (Biochrom) and 0.5 µg ml^{-1}

partricin (Biochrom) at 37°C in a humidified atmosphere with 95% air and 5% CO_2. These cells do not grow to confluency, and were thus split 1:5 every four days when they reached approximately 70–80% confluency. Splitting was done through digestion of cells in PBS with 0.05%/0.02% trypsin/EDTA (w/v).

Serum used for cell culture contains steroid hormones. This can interfere with assays that determine steroid hormone receptor function, such as GR. FCS was stripped using the charcoal method, and cell culture medium was supplemented with stripped FCS where indicated. To produce stripped FCS, 25 g of the anion exchanger resin AG1–X8 was stirred ON with 500 ml FCS. After settling of the resin, supernatant was decanted and mixed with 10 ml charcoal/dextran suspension and stirred again ON at 4°C. The suspension was spun down for 10 min at 1000g, and the supernatant was sterile filtered, followed by storage at –20°C until use.

Primary Cultures Of Human Corticotroph Adenomas And Normal Rat Pituitary Cells

Corticotroph pituitary adenomas were obtained from patients with Cushing's disease that underwent transphenoidal surgery. Adenomateous tissue was washed with HDB buffer and mechanistically dispersed into small fragments, followed by enzymatic dispersion for 45 min at 37°C in solution containing 4 g l^{-1} collagenase, 0.01 g l^{-1} DNAse II, 0.1 g l^{-1} soybean trypsin inhibitor and 1 g l^{-1} hyaluronidase II. Cell viability was determined by acridine orange/ethidium bromide staining, and only cultures with viability above 90% were considered and grown in DMEM (Gibco 95530) supplemented with 10% stripped FCS and antibiotics for 48 h prior to treatment in 96–well plates. Treatment was done in cell culture medium with 0.1% stripped FCS. All experiments with human material were performed after approval of the local ethics committee of the Ludwig Maximilian University of Munich – Germany, and informed written consent was received from each patient whose pituitary adenoma tissue was used in the study.

Primary cultures of normal pituitary was produced from rats. The size and architecture of the rat pituitary allows for dissection of the anterior pituitary from the intermediate lobe, the latter of which contains cells that produce ACTH as well and which could interfere with the measurements aimed to investigate the function of anterior pituitary corticotroph cells. Male Sprague–Dawley rats (at 6–7 weeks of age) were allowed to acclimate for 4 days at the Institute's animal facility before sedation with CO_2, decapitation and dissection of the anterior pituitary. Enzymatic dispersion of isolated pituitary was done as described above for pituitary

adenomas, and cells were seeded at a densitiy of 2 x 10^4 cells per well in 96–wells in cell culture medium containing 10% stripped FCS and vitamins for 48 h. Treatment was done in cell culture medium with 1% stripped FCS plus MEM vitamins.

WST–1 Cell Viability Assay

To assess the viability of cells under different treatment conditions, the WST–1 assay was used. This ready to use assay mixture contains tetrazolium salt, which is cleaved to soluble formazan in a cellular process which depends on the glycolytic production of NAD(P)H in mitochondria of cells, and the amount of formazan dye formed directly correlates with metabolically active (= viable) cells in the culture.

AtT–20 cells in cell culture medium were seeded at 2 x 10^3 cell per well in 96–well plates and left to attach for 24 h. Medium was changed to cell culture medium with 2% FCS and drugs were added for 96 h at the indicated concentrations with a constant DMSO concentration of 0.2%. After drug incubation the medium was quickly replaced by DMEM with WST–1 substrate according to the manufacturer's instructions. After 30 min, the absorption was measured at 450 nm in a plate reader. Graphs were done using SigmaPlot v12.5.

FACS Analysis

FACS was conducted to specifiy the effects of the small molecule inhibitors on the cell cycle. Fixation of cells with EtOH and staining with the fluorescent dye propidium iodide is a reliable and efficient method to determine the DNA content of cell populations. Propidium iodide intercalates with DNA in the nucleus of each cell. The cell sorter counts single cells in solution, forced in a stream through a thin capillary. Identification of single cells is made possible through a transparent section inside the capillary: the cell sorter sends light through that section when the cells are passed through at a constant pace and detects A) the forward scatter (FS) and the side scatter (SS), and subsequently B) a specific fluorescent signal. FS identifies the volume of a cell (important if, for example, plasma cell populations are investigated). SS identifies the quantitiy and characteristics of intracellular structures such as granularity, size of the nuclei etc. Figure M1a shows a SS/FS plot used in this work. Since the AtT–20 cell line was used, the readout depicts a homogenous cell population. The selection lines are set as a first threshhold for further analysis: unwanted artifacts such as debris (low signal) or very large aggregates (high signal) are thus excluded (**Fig. M1a**). Next, the fluorescent signal gained through propidium idodide excitation is processed. Figure M1b shows that the fluorescence signal gives 3 distinct outcomes for diploid cells (2n – G1 phase

of the cell cycle), tetraploid cells (4n – G2/M phase) and, for example, a doublet of two diploid cells sticking together (which is an artifact caused by cell preparation). The intensitiy of the fluorescent signal directly correlates with the DNA content of a cell (peak H), i.e. the peak for cells in S–phase lies between those obtained for 2n and 4n. The area under the curve (integral I) is the second parameter which allows to exclude doublets, since H = 1 for both a single diploid cell and for a doublet of two diploid cells (**Fig. M1b**). Counting doublets as one diploid cell would skew the result towards an underestimation of the relative amount of diploid cells. Figure M1c shows a plot which incorporates H over I, and a selection for apoptotic and single cells only (**Fig. M1c**). Figure M1d shows the cell cycle distribution: the number of events for each H are plotted, and cell cycle subpopulations are designated according to the DNA content of each event (subG1 – hypodiploid cells equal apoptotic cells). The graph calculates the percentage of 20.000 events which passed all gating criteria according to the thresholds (**Fig. M1d**). Using this process, the results in the work presented here were generated. Bar charts were done using Excel, and histograms with the EPICS System II Software.

Figure M1

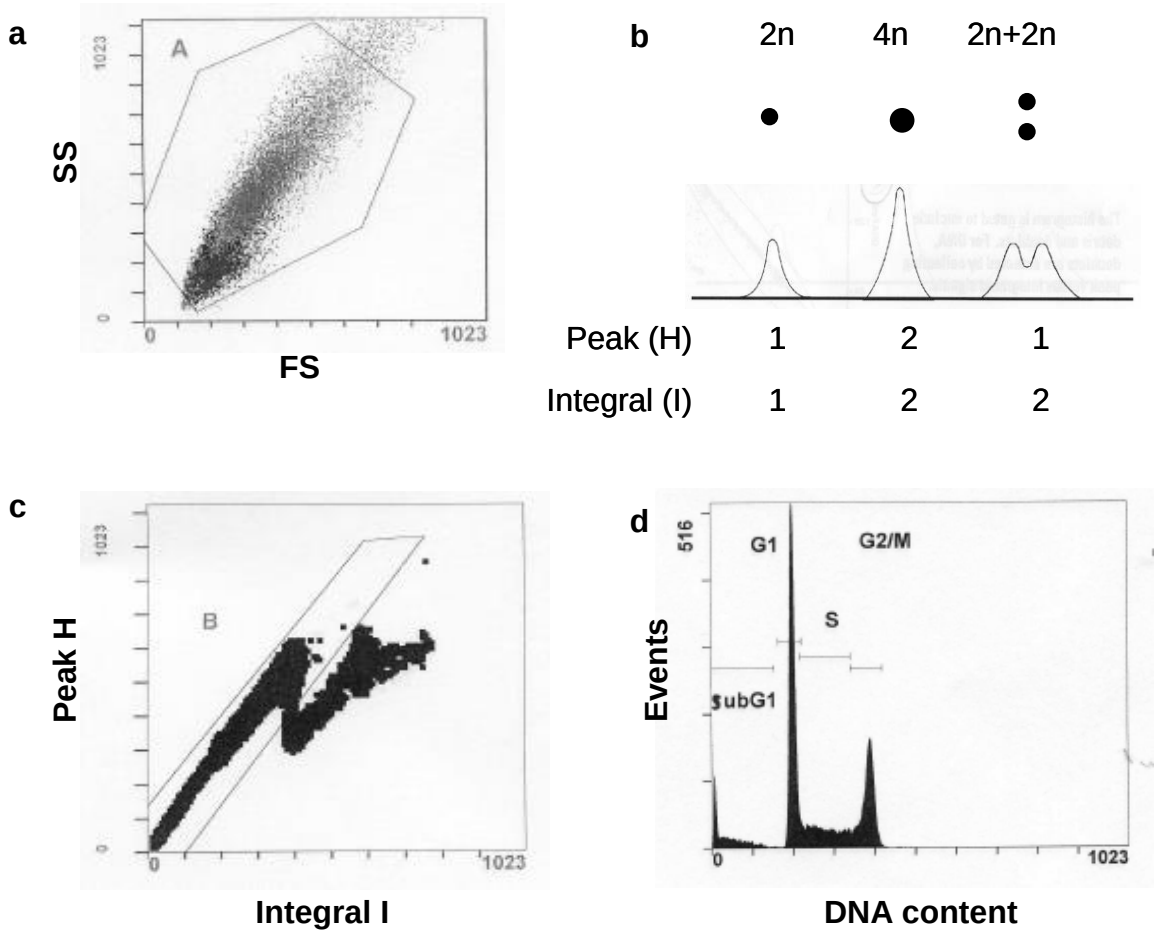

Figure M1: Step by step schematic representation of FACS analyses conducted in the experiments of the present work.

AtT–20 cells in cell culture medium were seeded at 3 x 10^5 cells per well in 6–well plates and left to attach for 24 h. Medium was changed to cell culture medium with 2% FCS and the cells were incubated with the indicated drugs over time. DMSO was kept constant at 0.2% for all conditions. After incubation, cell supernatant was collected. The cells were washed in PBS and trypsinized in 1 ml per well for 4 min. The detached cell suspension was harvested into 8 ml cell culture medium and pelleted at 600g together with the supernatant, followed by a wash in 10 ml cold PBS. The pellet was redissolved in 1 ml cold PBS and pure ethanol was added dropwise to a final concentration of 75% under slight vortexing. The cells were fixed for 24 h at –20°C, washed in 1 ml cold PBS and the pellet was redissolved in 500 µl PBS with 10 µg ml^{-1} propidium iodide and 1 µg ml^{-1} RNAse A for 1 h at room temperature, followed by FACS in a Beckman–Coulter EPICS XL.

IMMUNOBLOT

The Western Blot is an invaluable molecular biological method which allows to determine the cellular content of specific proteins, and likewise, the influence of a vast number of conditions on these protein levels. Electrophoretic seperation according to the size of the proteins is followed by a transfer onto a sticky membrane which unspecifically binds protein. After blocking of the membrane at spots where no protein was transferred, specific antibodies are incubated which recognize the protein of interest. A secondary antibody coupled to HRP which recognizes the Fc–region of the primary antibody is incubated, and after washing, ECL substrate is added to the membrane, resulting in a light reaction. The light reaction is detected by a film, and the amount of protein correlates with the signal intensity, thus allowing for quantification.

Total cellular protein, called the lysate, is mixed with sample buffer containing SDS and a reducing agent, mostly didthiothreitol (DTT) or β–mercaptoethanol. Cooking of the lysate with sample buffer leads to breakage of disulfide bonds in the peptide chain, destroying the secondary structure. The now linear polypeptide chain is equally covered by SDS, resulting in a net negative charge of the protein which correlates with its length. The mix is applied onto a polyacrylamide gel with varying pore sizes, depending on the percentage of acrylamide used for preparation. The stacking gel with the wells where the mix is filled into has a pH of 8.8. The running buffer contains glycine, which is zwitterionic at pH 8.8, building an ion–border towards the cathode. The proteins are sandwiched between glycine and the negatively charged

chloride of the running buffer on their way to the anode, resulting in concentrated thin layers of protein prior to their entering into the seperation gel. As soon as the proteins enter the seperation gel, which is at pH 6.8, the glycine is negatively charged and passes the proteins, thus allowing for seperation of proteins according to their size. After electrophoresis, the proteins are transferred onto a PVDF membrane in an electric field in transfer buffer according to Towbin, where specific proteins can be detected using antibodies.

AtT–20 cells in cell culture medium were seeded at 3 x 10^5 cells per well in 6–well plates and left to attach for 24 h. Medium was changed to cell culture medium with 2% FCS and the cells were incubated with the indicated drugs over time. DMSO was kept constant at 0.2% for all conditions. After incubation, the cells were washed in cold PBS, and scraped in 150 µl RIPA buffer per well with protease inhibitor cocktail. The crude lysate was frozen at –80°C until use. After rapid thawing in a 37°C waterbath, the crude lysate was passed 10x through an insulin needle, followed by 15 min centrifugation at 16.000g and 4°C. The supernatant was used and is referred to as lysate. The protein content of each lysate was determined using the Bradford method according to the manufacturer's protocol. BSA was used as standard. The standard curve was determined for 25, 20, 10, 5 and 0 µg ml^{-1} of BSA. The Bradford reactive was diluted 1:1 in DDW and the samples were diluted as much as needed so that the values of the protein concentration were into the range of the standard curve. 100 µl of each standard and each sample were put in a transparent 96–well plate together with 50 µl of Bradford reactive. Absorbance was measured in a plate reader at 595 nm. Between 10 and 30 µg of total protein was mixed with 4x sample buffer (RotiLoad I) to a final concentration of 1x and boiled for 5 min at 95°C, followed by SDS-PAGE and transfer onto a PVDF membrane at constant 25V over night at 4°C in transfer buffer. The membrane was soaked in 100% methanol, air dried, followed by blocking in TBST with 5% skim milk for 1 hour at room temperature and incubation with primary antibody over night at 4°C, 3 x 5 min washing in TBST, and one hour incubation with secondary antibody at room temperature, 3 x washing for 5 min in TBST. Signal was obtained through incubation with ECL substrate and detection by a light sensitive film.

HSP90–CTD BINDING ASSAY

In order to investigate the binding site of Silibinin in Hsp90, a binding assay for small molecule compounds was developed. Morra et al. have demonstrated that chemical compounds can be immobilized to a polystyrene surface in the 96–well plate format, followed by incubation with the recombinant C–terminal domain of Hsp90 to allow for binding. The

bound protein was detected with antibodies, thus, this assay can principally be considered as a modified enzyme linked immunosorbent assay (Morra et al., 2010).

The cloning of amino acids 566 to 732 of human Hsp90α (Hsp90–CTD) into pPROEXHTa was described previously (Young et al., 2003). The expression plasmid was a gift from Dr. Hartl (MPI of Biochemistry) to Dr. Hausch (MPI of Psychiatry). Expression in *E. coli* BL21 was induced with IPTG (Sigma) and the His-tagged recombinant protein was purified using an Ni–NTA column (Quiagen), followed by dialysis into HG buffer to a final concentration of 5 mg protein per ml. Aliquots of Hsp90–CTD were a gift from Dr. Hausch.

To immobilize Silibinin to the polystyrene surface, it was dissolved in pH 9.5 bicarbonate buffer (BCB) with a final DMSO concentration of 5% and incubated ON at 4°C in untreated polystyrene microtiter plates (Costar 3695 – Corning). After a rapid wash in PBS, the wells were blocked in PBS with 1% gelatine for 1 h at RT. Recombinant human Hsp90–CTD was diluted in PBS with 1% BSA and incubated for 1 h at RT to allow for binding to immobilized Silibinin. After three washes in PBS, the wells were incubated for 1 h at RT with antibody directed against the C–terminus of Hsp90 (clone 68/Hsp90 1:1500 – BD Biosciences), diluted in PBS with 3% BSA and 0.1% Tween–20. After three washes in PBS, HRP–conjugated secondary antibody diluted in PBS with 3% BSA and 0.1% Tween–20 was added for 1 h, followed by three washes in PBS. Quantitative determination of bound Hsp90–CTD was done through the addition of the HRP–substrate reagent (DY999 – R&D), which contains tetramethylbenzidine (TMB) and H_2O_2. TMB acts as a hydrogen donor for the reduction of H_2O_2 to water, catalyzed by the HRP. After signal development, the reaction was stopped through the addition of 5N sulfuric acid, and the plates were read at 450 nm in a plate reader.

For competition assays, Hsp90–CTD bound to immobilized Silibinin was challenged with Silibinin or Novobiocin in solution for 1 h at RT. Silibinin was dissolved to a final concentration of 1 mM in PBS with 1% BSA, 0.05% Triton X–100 and 5% DMSO. Novobiocin was dissolved to a final concentration of 6 mM in TBS with 0.05% Tween–20 and constant 5% DMSO. All dilution series were done in the respective buffers. After three washes in PBS, the remaining bound Hsp90–CTD was detected with antibodies as described above.

CO–IMMUNOPRECIPITATION OF THE GR::HSP90 COMPLEX

Co–immunoprecipitation (CoIP) studies allow for the detection of protein–protein interactions. The isolation of protein I will result in detection of protein II, if they interact. Cellular lysate is incubated with anti–I antibody, and antibody is precipitated with magnetic

beads that are coated with recombinant protein G. Protein G is a high affinity binder of the immunoglobulin Fc–region. Precipitated complexes are attached to a magnet, which attracts the beads and allows for washing of the complexes. The complexes are then processed as for immunblot. Protein I and protein II are probed after SDS–PAGE and transfer onto a membrane. Using this method, it is possible to study whether two proteins interact with each other, and whether chemical compounds do influence this interaction.

AtT–20 cells in culture were grown at 1 x 10^6 per 10 cm dish for 48 h in cell culture medium, and an additional 48 h in cell culture medium with 2% FCS, washed and scraped in cold PBS. The cell pellets were pooled. Per dish, the cell pellet was dissolved in 150 µl TEDGM supplemented with complete protease inhibitor and sonicated twice with a microtip for 1 s at intermediate setting, followed by centrifugation for 15 min at 16.000g and 4°C. The supernatant was precleared with 10 µl per dish Dynabeads Protein G for 30 min rotating at 4°C. Aliquots of the supernatant were incubated with increasing concentrations of Novobiocin dissolved in TEDGM with 0.1% Triton X–100 or Silibinin dissolved in TEDGM with 1% BSA and 0.05% Igepal–630 with constant 3% DMSO for 1 h at 4°C. Prior to drug incubation, the aliquots of the lysate were adjusted to 0.1% Triton X–100 for Novobiocin and 0.5% BSA and 0.05% Igepal–630 for Silibinin. The different compositions of the lysates did not significantly influence yield or unspecific precipitation as determined by a non–antibody control as well as rabbit IgG non–immune antibody. 2 µl per dish H–300 or 0.4 µg per dish PA3–013 antibody was added for 2 hours at 4°C. The immune complex was captured with 10 µl per dish Dynabeads Protein G for 30 min at 4°C and washed 3 times with TEDGM with 0.1% Tween–20, and once with TEDGM without detergent, followed by boiling in sample buffer and immunoblot. On the membrane, GR was detected with M–20 antibody, and Hsp90 with H90–10 antibody.

^{3}H–Dexamethasone Binding To GR In AtT–20 Cells

AtT–20 cells in cell culture medium with 10% stripped FCS were seeded at 2 x 10^5 cells per well into 24–well plates and left to attach for 24 h. The medium was changed to cell culture medium with 0,1% stripped FCS and Silibinin was added at a concentration of 30 µM for 48 h. Vehicle was kept at 0,2% DMSO. The medium was replaced by DMEM with increasing concentrations of tritiated Dexamethasone (^{3}H–Dexamethasone) with or without a 500–fold excess of unlabelled Dexamethasone and incubated for 4 hours at 4°C. The ethanol concentration was kept constant at 0,5%. The cells were washed three times for five minutes with 500 µl cold PBS at 4°C. The cells were then lysed in 150 µl Passive Lysis buffer per

well, and the lysate was added to 3 ml scintillation fluid, vortexed an counted in a beta counter (Beckman LS6000IC). In parallel, control wells were trypsinized and counted. The data shown refers to counts per minute (cpm) per 1 mio cells for the Silibinin experiment, while in the 17–AAG experiment, the values represent cpm per well. Specific binding was calculated by subtraction of unspecific binding (in the presence of excess unlabelled Dexamethasone) from total binding. Maximal specific binding (B_{max}) and the dissociation constant (K_d) was determined using nonlinear regression for single site saturation ligand binding as implemented in SigmaPlot. GR binding sites per cell were calculated from B_{max} and the standard curve for input of ^{3}H–Dexamethasone. The Scatchard plot was derived from specific binding divided by input (bound/free) plotted over specific binding. The values depicted for competition of ^{3}H–Dexamethasone to GR with increasing concentrations of unlabelled Dexamethasone or the GR antagonist RU–38486 represent total binding.

REPORTER ASSAYS

Reporter gene assays investigate the transcriptional activity of a specific promoter region. It can provide the researcher with direct conclusions on the activity of the corresponding endogenous promoter, and thus, the mRNA produced by the downstream gene. As can be the case for quantitative RT–PCR, however, mRNA stability of the endogenous downstream gene does not play a role in reporter assays, since the promoter under investigation is fused to a reporter gene which introduces a 'standardized' mRNA. These assays can be a convenient tool to identify certain transcription factor response elements, and to study the impact of mutations thereof. It can be cotransfected with expression vectors for transcription factors of interest, dominant negative mutants thereof, or for cellular inhibitors of transcription factors. In the present study, reporter assays were used to determine the effects of endogenous or overexpressed GR on promoter activity under the influence of the Hsp90 inhibitors.

The MMTV–Luc construct contains four GR response elements which are naturally contained in the long-terminal repeat sequence of the mouse mammary tumor virus (MMTV) (Hollenberg and Evans, 1988), fused to the luciferase reporter gene. This construct constitutes a very strong promoter for GR mediated transcriptional effects, and has been extensively used to study GR function. The TK–Luc is a minimal promoter (tyrosine kinase) fused to the luciferase promoter. The GRE_2–TK-Luc contains two artificial consensus GR response elements (GRE) integrated upsteam of the TK minimal promoter (Rupprecht et al., 1993). Since this is an artificial construct with the GREs being the only regulatory binding sites, it can be used to study direct effects of GR without the influence of other (transcription) factors.

The POMC–Luc construct contains the 770 base pairs rat POMC promoter and includes all the sequences necessary for the expression and regulation of POMC in corticotroph cells (Liu et al., 1992). It was used here to investigate the role of GR as trans–repressor of Nur77 (Philips et al., 1997b). The NurRE-Luc is a reporter which contains three copies of a Nur response element fused upstream of the POMC minimal promoter (Philips et al., 1997b), and this construct was used to confirm the results for POMC–Luc. The amount of luciferase transcribed from any reporter gene correlates with the conversion of luciferin to produce light during the measurement of cellular lysate in a chemiluminescent plate reader. In RSV–Gal, the Rous–Sarcoma Virus (RSV) promoter is a strong constitutive promoter which displays relatively little reactivity on a number of cellular stimuli. It is thus used as a control reporter to adjust for transfection efficiency between wells. The galactosidase reporter activity is measured by colorimetry at 405 nm after the enzyme's conversion of synthetic o–Nitrophenyl–β–D–galactopyranosid into galactose and the yellowish o–Nitrophenol. In all experiments, the luciferase values were divided by the galacatosidase values.

AtT–20 cells in cell culture medium with 10% stripped FCS were seeded at 1 x 10^5 cells per well into 24–well plates and left to attach for 24 h. Per well, 0.4 µg empty pcDNA3.1+ (Invitrogen), 0.3 µg reporter plasmid and 0.1 µg RSV–Gal were mixed with 40 µl DMEM and 4 µl Superfect reagent. After 10 min at RT, 210 µl cell culture medium without FCS were added, and after washing the cells with PBS, 250 µl transfection mix was added for 3 h. The transfection mix was then replaced with cell culture medium without FCS ON. Treatment of the cells was done in cell culture medium with 2% stripped FCS and a constant DMSO concentration of 0.2%. After drug incubation, the cells were washed in cold PBS, and lysed in 150 µl Passive Lysis buffer ON in –80°C. For galactosidase activity, 20 µl lysate was added to 30 µl aqua bidest. and mixed with 50 µl 2x ONPG buffer, followed by incubation at 37°C and measurement at 405 nm in a plate reader. For luciferase activity, 20 µl lysate were mixed with 50 µl luciferin in the TriStar (Berthold), followed by measurement of chemiluminescence. The final results are the ratio of luciferase/galactosidase to control for transfection efficiency between the wells. Graphs were done using Microsoft Excel.

MUTAGENESIS

We received the expression vector for rat GR from Dr. Simons Jr. (NIH – Bethesda, USA). To produce the P548A/T549A/V551A triple mutant (Kaul et al., 2002), the pSVLGR–WT plasmid was used for single–step mutagenesis with the QuikChange II XL site–directed Mutagenesis Kit. The forward mutagenesis primer was 5'–CCA CAG CTC ACC GCT GCC

TTG GCG TCA CTG–3', and the reverse mutagenesis primer had the sequence 5'–CAG TGA CGC CAA GGC AGC GGT GAG CTG TGG–3', with the mutated nucleotides underlined, and as depicted in the table.

base pair (position)					C/G(1652)	A/G(1655)		T/C(1662)		
sequence	CCA	CAG	CTC	ACC	**G**CT	**G**CC	TTG	G**C**G	TCA	TCG
amino acid position	P544	Q545	L546	T547	**P548A**	**T549A**	L550	**V551A**	S552	L553

Table M1: Summary of the mutagenesis.

To introduce the mutations, 10 ng plasmid DNA was mixed 125 ng forward and reverse primer each, plus PCR reactants according to the manufacturer's instructions. An initial cycle at 95°C for 30 min was followed by 18 cycles of: 95°C for 30 s; 55°C for 1 min; 68°C for 7.5 min. Thereafter, 1 µl of DpnI endonuclease was incubated for 1 h at 37°C. DpnI cleaves 5'–GA^TC–3' only when the adenosine is methylated. Thus, the plasmid that was propagated in bacteria, and which is used for mutagenesis, will be digested. The linear, mutated amplicons will be preserved for further processing.

The digested DNA (1 µl) was added to 50 µl *E. coli* XL1–blue and incubated on ice for 30 min, followed by 45 s in a 42°C waterbath, and again 2 min on ice. 1 ml prewarmed NYZ+ medium was added, and the culture was put on a shaker for 1 h at 37°C (included in the kit). Then, 250 µl culture were plated on LB agar plates containing 50 µg ml^{-1} Ampicillin ON.

To confirm successful mutagenesis, 6 clones were picked for propagation ON in LB medium with 50 µg ml^{-1} Ampicillin, and plasmids were purified using the Qiagen Mini–Prep kit. The plasmids were sent for sequencing at SequiServe (Vaterstetten, Germany), and all clones showed the desired base pair exchanges at positions 1652, 1655 and 1662 in comparison to the wildtype (WT) plasmid.

For reporter gene assays with overexpressed GR, AtT–20 cells were transfected with 0.2 µg expression plasmid (WT or mutant), 0.2 µg pcDNA3.1+, and reporter as described above. Nonlinear regression was done using SigmaPlot. Treatment was done as described above.

IMMUNOCYTOCHEMISTRY

In order to investigate the subcellular localization of GR and Hsp90 upon treatment with the Hsp90 inhibitors and/or Dexamethasone, culture slides of AtT–20 cells were used for immunocytochemistry (ICC). 1 x 10^4 cells per well in cell culture medium were seeded in a 8–chamber slide and left to attach for 24 h. After that, medium was changed to cell culture medium with 0.1% FCS and cells were treated for 48 h with Silibinin or Novobiocin, or 15

min with 17–AAG. The DMSO concentration was kept constant at 0.2%. Dex was added for the last 15 min. The medium was removed and the cells were fixed for 10 min at room temperature with PBS and 4% PFA (prepared freshly). After three washes in PBS, cells were permeabilized for 10 min at RT with 0.2% Triton X–100 in PBS, followed by blocking for 1 h at RT with PBS and 3% BSA. Antibody to GR (H–300 1:500) and antibody to Hsp90 (H90–10 1:500) were diluted in blocking solution with 0.1% Tween–20 and incubated over night at 4°C. After 3 washes in PBS, fluorophore conjugated secondary antibodies were diluted 1:500 in blocking solution with 0.1% Tween–20 and incubated for 1 h at RT, followed by 3 washes in PBS. Cells were mounted in ProLong® antifade Gold which contains DAPI to counterstain for DNA, and covered with a cover slip. Slides were evaluated in a Zeiss Axioskop II connected to a mercury lamp through filters according to the fluorophores.

RADIOIMMUNOSSAY OF ACTH

In order to investigate ACTH secretion from AtT–20 cells and primary cultures of human corticotroph adenomas as well as normal rat pituitary cells, the ACTH radioimmunoassay (RIA) was performed. The assay procedure was modified in our laboratory and utilized in several published works (Paez-Pereda et al., 2001; Labeur et al., 2008; Giacomini et al., 2006; Reiter et al., 2011). ACTH standard is labeled with radioactive iodine by Chloramin T reaction. This method incorporates ^{125}I into the single tyrosine residue in ACTH. The principle of this assay is based on the competition of ACTH in cell culture supernatant with a fixed concentration of labeled ACTH for an antibody directed against ACTH. The more ACTH in the cell supernatant, the less labeled ACTH binds to the fixed concentration of antibody. A secondary antibody is bound to the primary antibody to increase immunglobulin complex size. This large complex can be precipitated using polyethylene glycol buffer to separate specific residual ^{125}I–ACTH bound to the antibody–complex from unbound ^{125}I–ACTH in solution. Radioactivity is measured in a gamma counter. A standard curve is included in each experiment, and an initial determination of the ACTH concentration in the samples is used to calculate the sample dilution range in order to be in the linear range of the standard curve. For labeling, ACTH–standard was diluted to 1 mg ml^{-1} in 0.01 M acidic acid and diluted 1:10 with phosphate buffer. ^{125}I in phosphate buffer was mixed with Chloramin T and the reaction was stopped after 5 s with sodium disulfite. After seperation of labeled ACTH with silica gel, washing and elution, the ^{125}I–ACTH stock (tracer) was frozen until further use. The standard curve was produced through serial dilutions of unlabeled ACTH (1 mg ml^{-1}) in DMEM to 10.000 – 5000 – 2500 – 1250 – 625 – 312 – 156 – 78 – 0 pg ml^{-1} and

incubation ON at 4°C with 20.000 – 25.000 cpm per 100 µl tracer and primary antibody directed against ACTH (in–house production). Secondary antibody (anti–rabbit IgG) was added for 1 h at RT. The complex was isolated with PEG buffer, and the pellet was counted in a Wizard 1470 gamma counter (Perkin Elmer). Figure M2 shows a typical standard curve. The abscissa depicts the concentration of the standard. On the ordinate, B/T stands for bound divided by total and is calculated for each value by the measurement of radioactivity for each concentration of standard used, minus the unspecific signal (primary antibody was omitted) – the value 0.1 represents 100% binding of tracer for 0 pg ml^{-1} standard, and the binding signal decreases with increasing concentrations of standard. The sample values calculated from the fitted regression curve correspond to pg ml^{-1}.

Figure M2

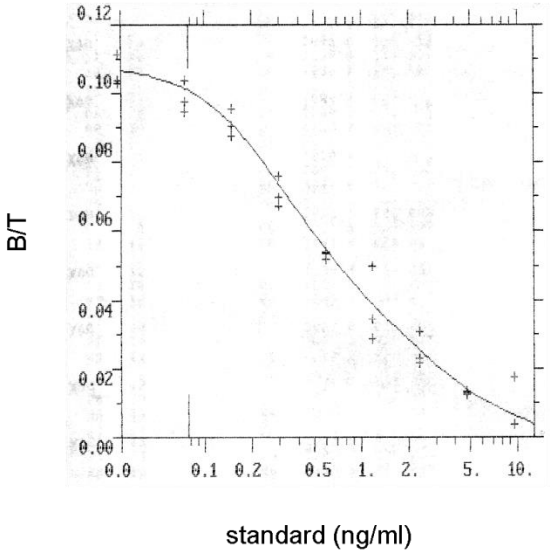

Figure M2: Representative example of the standard curve used in the RIA experiments.

AtT–20 cells in stripped cell culture medium were seeded at 1 x 10^5 cells per well into 24–well plates and left to attach for 24 h. Cells were treated with Silibinin or 17–AAG for 24 h in cell culture medium with 0.1% stripped FCS. Thereafter, cells were washed in DMEM and treated for an additional 24 h with Silibinin or 17–AAG, and Dexamethasone in cell culture medium with 0.1% stripped FCS, and the supernatant was frozen for RIA. DMSO was kept constant at 0.2% for all conditions.

After attachment, primary cultures of human corticotroph adenomas and normal rat pituitary cells were treated identical to AtT–20 cells as described above, except that the medium for rat normal pituiatry cells contained 1% stripped FCS and MEM vitamins.

AtT–20 Allograft Model

The Max Planck Institute of Psychiatry does not have the facilities to house immunodeficient mice. The animal studies, except for hormone measurement, were conducted by an external company. Immunodeficient male NMRI nude mice were injected unilateral subcutaneously with 30 µl AtT–20 cells dissolved in PBS at a concentration of 5 x 10^6 per ml. After seven days, animals were randomized into vehicle (n = 12; 10 ml kg^{-1} day^{-1} aqua bidest.) and treatment group (n = 12; 300 mg kg^{-1} day^{-1} Silibinin (Silicur – Hexal) in aqua bidest.). Silibinin or vehicle was administered intraorally. Tumor volumes were determined every four days with a calliper. Blood samples were collected by cardiac puncture 24 h after the last treatment from isoflurane–anesthetized animals.

RIA was utilized to measure plasma ACTH (ImmuChem™ from MP Biomedicals) according to the manufacturer's instructions, as well as plasma corticosterone (Rat/Mouse RIA from DRG Instruments). All animal experiments were conducted according to the guidelines of the German Animal Welfare Act.

Statistical Analysis

$P<0.05$ was considered statistically significant in two–tailed, unpaired Student's *t* tests or Welch's *t* test, or repeated measures ANOVA. For the allograft model in nude mice, the sample size of 12 animals was chosen because a strong effect size for the treatment on tumor growth and hormonal control was expected. Violations of parametric *t* test assumptions with 5% significance threshold were tested. The Kolmogorov–Smirnov test was applied in order to analyze normality of data distribution in the samples, followed by f–testing to verify whether the assumptions of equal variances between groups was fulfilled.

6 RESULTS

HSP90α IS OVEREXPRESSED IN BIOPSY SPECIMENS OF HUMAN CORTICOTROPH ADENOMAS

Overexpression or increased activity of the Hsp90 chaperone machinery has been shown in numerous types of cancer. Since strongly reduced levels as well as overexpression of Hsp90 impede GR signalling, it was hypothesized that the high threshold of agonist necessary to activate GR in corticotroph pituitary adenomas might be caused by aberrant expression and/or activity of Hsp90. Thus, as a basis to investigate this hypothesis, the expression level of the inducible Hsp90α isoform was determined by immunohistochemistry in biopsy specimens of human pituitary adenomas from patients with Cushing's disease that have undergone transphenoidal surgery. For comparison, post–mortem normal human pituitary tissue was examined.

As shown in Figure 1, strong overexpression of Hsp90α was evident in corticotroph adenomas from patients with Cushing's disease (n = 12/14). A representative specimen for the strong, cytoplasmatic staining in the homogenous adenoma tissue is shown. The two remaining corticotroph adenoma specimens which were not scored as highly overexpressing Hsp90α had poorly conserved tissue structure, and were thus excluded. In contrast, the normal human pituitary tissue showed a typical, heterogeneous morphology with only single cells expressing Hsp90α at a detectable level (n = 6/6). These can be identified as corticotroph cells by their morphology, and they expressed Hsp90α at a much lower level than the adenomateous cells do. To address the question if overexpression of Hsp90α is specific for corticotroph adenomas, its abundance was compared to that in hormone insufficient pituitary adenomas. Staining of 14 non–functioning pituitary adenomas was additionally conducted. No overexpression was found in this malignancy (n = 14/14). Of note, as compared to normal pituitary, no single cells with detectable Hsp90α expression interspersed in the homogenous adenoma tissue were observed (**Fig. 1**). Therefore, Hsp90α overexpression was specifically found in corticotroph adenomas.

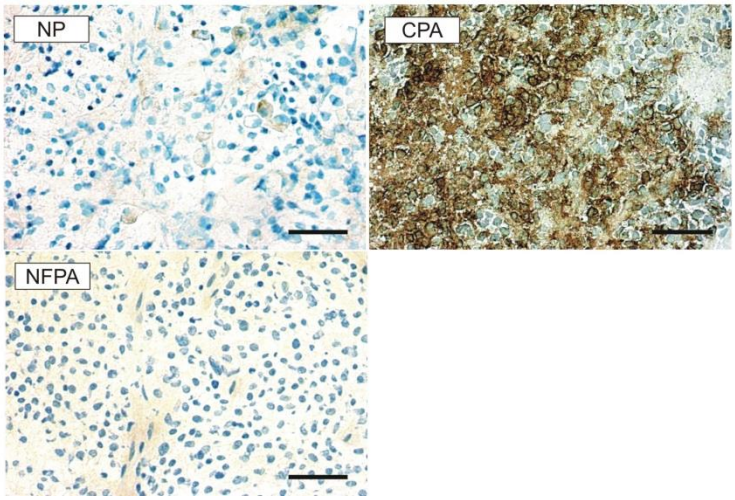

Figure 1: **Immunohistochemistry of Hsp90α in cryosections.** A representative example of a human corticotroph adenoma (CPA) from a patient with Cushing's disease, a non–functioning pituitary adenoma (NFPA) from a patient with hormone insufficiency, or post–mortem normal human pituitary tissue (NP) is shown. Counterstaining for nuclei was done with toluidine blue, the Hsp90α signal is brown (DAB). Scale bars: 40µm.

The Inhibition Of Hsp90 With Small Molecules Has Antiproliferative Effects In AtT–20 Cells

In order to investigate the role of Hsp90 in corticotroph adenoma function, the well established corticotroph adenoma cell line AtT–20 was established. This cell line is derived from a mouse corticotroph adenoma, and it is the only available cellular model for Cushing's disease – no human cell line is available. AtT–20 cells exhibit all endocrine features necessary to study the regulation of ACTH production: As in normal corticotroph cells, POMC is under control of the pituitary specific transcription factor Tpit, which acts in concert with Pitx (Lamolet et al., 2001). ACTH production is mainly enhanced by CRH, via downstream induction and activation of Nur77 (Kovalovsky et al., 2002). Basal and cAMP–induced ACTH–production is repressed through activated GR (Philips et al., 1997a; Philips et al., 1997b). Thus, AtT–20 cells retain the status of differentiated corticotroph cells, however, their malignant characteristics can be exploited to investigate aspects of cell cycle control which are deregulated in corticotroph adenomas.

To this end, three distinct Hsp90 inhibitors were chosen: the Geldanamycin derivative 17–AAG, which targets the N–terminal ATPase domain of Hsp90. The C–terminal Hsp90 inhibitor Novobiocin, and the recently discovered Hsp90 inhibitor Silibinin. As shown in Figure 2, the proliferation of AtT–20 cells is reduced in a dose dependent manner by each of the inhibitors after 96 h in culture (**Fig. 2**).

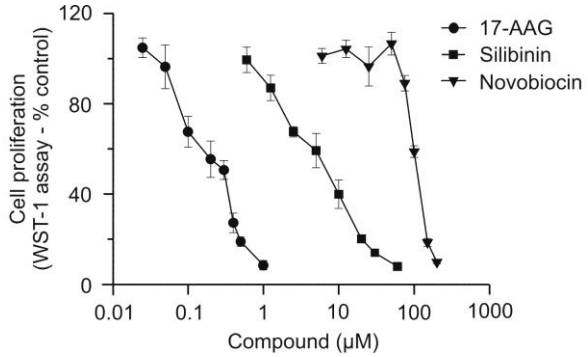

Figure 2: **Antiproliferative effects of Hsp90 inhibitors**. AtT–20 cells in culture were incubated with increasing concentrations of 17–AAG, Novobiocin, or Silibinin for 96 h, followed by determination of cell viability with the WST–1 assay. Shown are means ± s.d. Similar results were obtained for two replications of the experiments.

DIFFERENTIAL EFFECTS OF HSP90 THE INHIBITORS ON CELL CYCLE

Hsp90 integrates numerous signalling cascades which control survival and proliferation. To elucidate the mechanism by which the Hsp90 inhibitors exert their antiproliferative effects on AtT–20 cells, the cell cycle distribution was investigated in the presence of the different Hsp90 inhibitors. 17–AAG increased the hypodiploid population in a time– and dose–dependent manner, which indicates apoptotic DNA fragmentation (**Fig. 3a**). In contrast, both Novobiocin (except for the highest dose) and Silibinin caused a pronounced arrest of AtT–20 cells in the G2/M checkpoint of the cell cycle at 24 h. However at 48 h, AtT–20 cells treated with Novobiocin show increased cell death, and the G2/M population is lost. The clinically safe Hsp90 inhibitor Silibinin led to sustained arrest of AtT–20 cells in the G2/M checkpoint of the cell cycle also after 48–hour treatment, while no increase of apoptotic cells was observed except for the highest concentration (**Fig. 3b** and **c**). Therefore, different Hsp90 inhibitors produce distinct cellular effects.

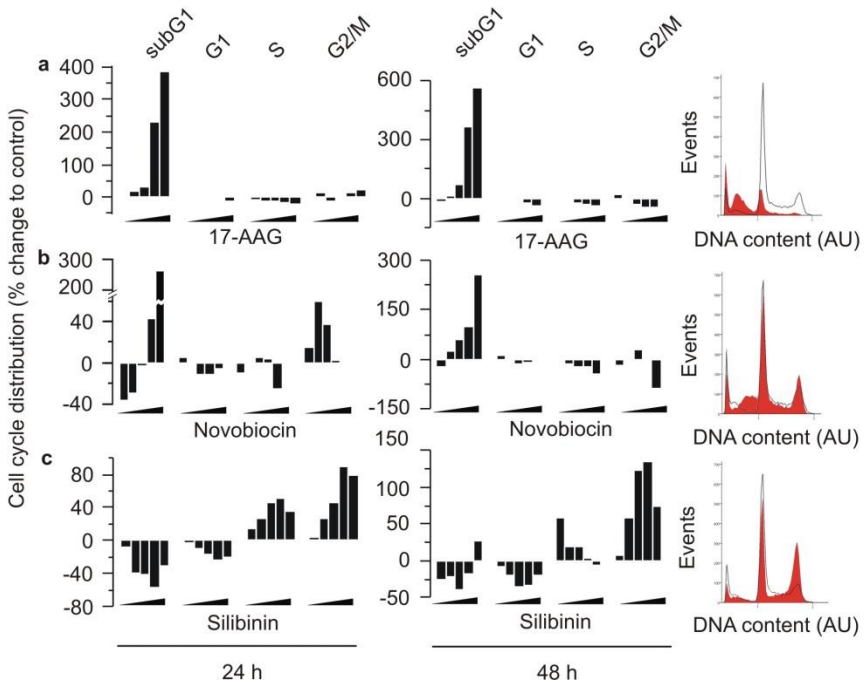

Figure 3: **Cell cycle distribution of AtT–20 cells in the presence of Hsp90 inhibitors**. Cell populations were determined by propidium staining and FACS analysis. The bar charts represent the percent change compared to control cells for increasing concentrations of each inhibitor. The bar charts and cell cycle profiles as depicted in the histogram to the right (□ control or ■ compound) were derived from raw data as described under Materials & Methods. (**a**) Effects of 17–AAG (0.25–0.5–1–1.5–3 µM) and after 48–hour treatment with 1.5 µM for the histogram. (**b**) Effects of Novobiocin (50–75–100–150–300 µM) and after 48–hour treatment with 150 µM for the histogram. (**c**) Effects of Silibinin (5–10–20–40–60 µM) and after 48–hour treatment with 40 µM for the histogram. The results shown are representative of three (17–AAG and Novobiocin) or four (Silibinin) independent experiments.

DIFFERENTIAL EFFECTS OF HSP90 THE INHIBITORS ON CLIENT PROTEIN STABILITY

A hallmark of the pharmacologic inhibition of Hsp90 is the degradation of client proteins. Due to the pronounced cell cycle arrest that is caused by the treatment of AtT–20 cells with Silibinin and Novobiocin, the cellular protein level of the central mediator of mitotic progression, Cdc2, was determined. Cdc2 is a prominent Hsp90 client protein (Garcia-Morales et al., 2007; Nakai and Ishikawa, 2001). As shown in Figure 4a, all inhibitors tested led to the degradation of Cdc2 with increasing concentrations after 48–hour treatment. The protein level of Hsp90α was not influenced by the compounds (**Fig. 4a**).

Due to the important physiological role that the GR plays in the suppression of ACTH in corticotroph cells, its protein level was investigated. As was shown for Geldanamycin (Whitesell and Cook, 1996; Segnitz and Gehring, 1997), the N–terminal Hsp90 inhibitor 17–AAG caused the degradation of GR. In sharp contrast, neither Novobiocin nor Silibinin influenced the cellular GR protein level. This is the first report that different Hsp90 inhibitors have diverging effects on the cellular fate of distinct classes of Hsp90 client proteins (**Fig. 4a**).

It was reported that Geldanamycin and derivates readily cross the cell membrane and that they show immediate effects of Hsp90 inhibition. Time course experiments were done to investigate when the inhibitors proved activity. As shown in Figure 4b, 17–AAG exhibited effects on the cellular Cdc2 and GR protein level already 3 h after the start of drug incubation. Novobiocin and Silibinin had detectable effects starting at 6 h, with strong effects from 12 h onwards. Again, neither Silibinin nor Novobiocin had an effect on GR protein levels at any time point (**Fig. 4b**).

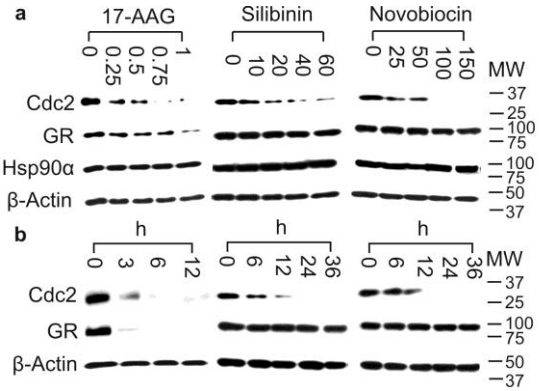

Figure 4: **Investigation of the effects of Hsp90 inhibitors on client protein stability.** (**a**) AtT–20 cells were treated for 48 h with the indicated Hsp90 inhibitors (μM), followed by SDS–PAGE and protein detection by immunoblot with antibodies to Cdc2, GR, Hsp90α, and β–Actin. (**b**) AtT–20 cells were treated with 5 μM 17–AAG, 200 μM Novobiocin or 60 μM Silibinin for the indicated periods of time, and cellular protein was determined as in **a**. Each experiment was repeated at least twice (17–AAG) or three times (Novobiocin and Silibinin), with similar outcomes.

SILIBININ BINDS TO THE C–TERMINAL DOMAIN OF HSP90 IN A REVERSIBLE MANNER

The observation that Silibinin and Novobiocin caused G2/M arrest and did not impact cellular GR protein levels at concentrations of active and specific Hsp90 inhibition – as shown by degradation of Cdc2 – led to the hypothesis that both inhibitors share the same molecular mechanism. Therefore, it is likely that they bind to the same domain in Hsp90. Due to the results obtained with trypsin nicking, protein truncation experiments and molecular modelling, it has been proposed that Novobiocin binds to a cavity in the C–terminal domain (CTD) of Hsp90 (Yun et al., 2004; Marcu et al., 2000a; Matts et al., 2011). However, it has never been shown that Silibinin binds to the CTD of Hsp90. To this end, the fact that small molecule compounds can be immobilized on a polystyrene surface to detect its interaction with purified recombinant Hsp90–CTD was exploited (Morra et al., 2010). As shown in Figure 5a, recombinant human Hsp90–CTD (amino acids 566–732 of human Hsp90α) (Young et al., 2003) could be detected after incubation on immobilized Silibinin. The binding curve followed a sigmoidal pattern, i.e. at the lowest concentrations minimal Hsp90 binding

was detectable, and the binding was saturated at the highest concentrations (**Fig. 5a**). Importantly, Hsp90–CTD bound to immobilized Silibinin could be challenged by excess Silibinin and Novobiocin in solution. Furthermore, the incubation of increasing concentrations of Hsp90–CTD with a constant concentration of immobilized Silibinin (64 µM) also resulted in a binding curve in which the input concentration highly correlated with the observed binding signal (**Fig. 5b**).

The highest concentration of Silibinin used in the competition assay did not completely reverse Hsp90–CTD binding. Higher concentrations precipitated after high speed centrifugation of the working solution, and resulted in irreproducible artifacts in the binding assay, namely a variable increase in Hsp90–CTD signal as opposed to specific competition of lower Silibinin concentrations (data not shown). In contrast, the formulation of Novobiocin used in this work (Novobiocin sodium salt) is water soluble, thus, solubility was not a limiting factor. The highest concentrations fully competed Hsp90–CTD binding to immobilized Silibinin (see Fig. 5a). Together, these results suggested that Silibinin and Novobiocin share an identical or overlapping binding motif on the CTD of Hsp90.

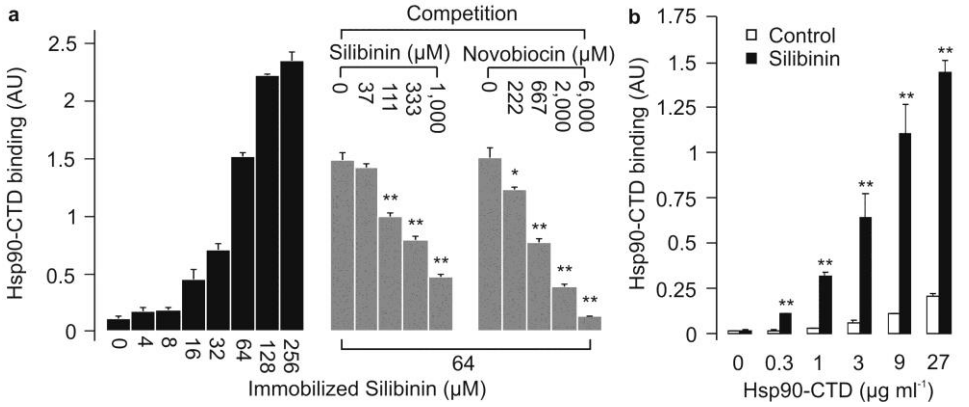

Figure 5: **Binding of recombinant human Hsp90–CTD to Silibinin.** (**a**) Increasing concentrations of Silibinin immobilized to a polystyrene surface were incubated with 3 µg ml^{-1} of Hsp90–CTD, followed by ELISA. Bound Hsp90–CTD was challenged with Silibinin or Novobiocin in solution. (**b**) 64 µM Silibinin were immobilized and incubated with increasing concentrations of Hsp90–CTD. Data in **a** and **b** show means ± s.d. of representative results of at least three independent experiments (*$P \leq 0.05$, **$P \leq 0.01$; compound vs. control).

THE C–TERMINAL HSP90 INHIBITORS SILIBININ AND NOVOBIOCIN DISSOCIATE THE GR::HSP90 COMPLEX IN VITRO

After binding, Novobiocin forces Hsp90 into a conformation that releases client proteins and leads, for example, to the dissociation of the client heme–regulated eIF2α kinase (Yun et al., 2004). Therefore, using co–immunoprecipitation, it was investigated here whether the C–terminal Hsp90 inhibitors Silibinin and Novobiocin dissociate the GR::Hsp90 complex in

AtT–20 cell lysates. It was found that either compound disrupted this complex in a concentration dependent manner, both when GR or Hsp90α were immunoprecipitated (**Fig. 6**).

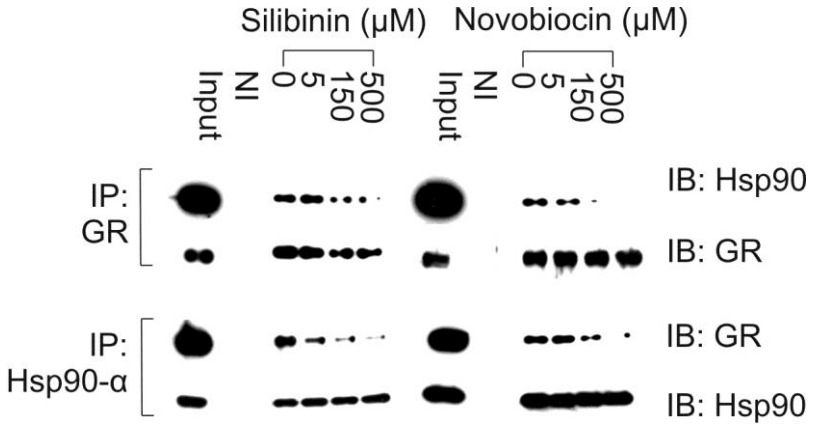

Figure 6: Effects of Silibinin and Novobiocin on the GR::Hsp90 complex. Cellular lysate of AtT–20 cells was incubated with the indicated concentrations of Hsp90 inhibitor at 4°C, followed by precipitation of GR or Hsp90α using specific antibodies (NI – non–immune rabbit IgG; IP – immunoprecipitation; IB – immunoblot).

SILIBININ INCREASES ^{3}H–DEXAMETHASONE BINDING TO GR IN AtT–20 CELLS

Earlier studies showed that GR requires dynamic interactions with Hsp90 to maintain its high affinity binding conformation (Dittmar et al., 1997), and that N–terminal Hsp90 inhibitors impair steroid binding (Whitesell and Cook, 1996; Segnitz and Gehring, 1997; Morishima et al., 2000). To determine whether GR loses steroid binding activity in the presence of Silibinin, we characterized its effects on ^{3}H–Dexamethasone binding to GR in AtT–20 cells. The cells were incubated with ^{3}H–Dexamethasone at 4°C following inhibitor treatment, since low temperature traps cellular processes, and it is thus possible to investigate the binding characteristics of GR at a particular moment (i.e. complexed to Hsp90 in the absence of inhibitor vs. dissociated from Hsp90 in the presence of inhibitor) without the influence cellular events which rapidly follow agonist binding (Galigniana et al., 2001; Czar et al., 1997). Unexpectedly, it was observed that Silibinin increased the number of GR sites (control: 36,240 ± 588 receptors per cell vs. Silibinin: 58,740 ± 1,855 receptors per cell) that bind ^{3}H–Dexamethasone with high affinity (control: K_d = 2.77 ± 0.37 nM vs. Silibinin: K_d = 2.49 ± 0.28 nM) (**Fig. 7a**), indicating that GR is fully mature after Silibinin treatment. Increasing concentrations of unlabeled Dexamethasone as well as the selective GR antagonist RU–38486 displaced the binding of ^{3}H–Dexamethasone (**Fig. 7b**). Furthermore, 15–minute treatment with 17–AAG prior to the addition of ^{3}H–Dexamethasone obliterated the high–affinity binding state of GR (**Fig. 7c**). This is in line with previous works that short term treatment

with Geldanamycin abolishes the steroid binding activity of GR prior to detectable degradation (Whitesell and Cook, 1996). The results show that the test system used here reflects specific binding of ^{3}H–Dexamethasone to GR in AtT–20 cells and the dependence of GR steroid binding activity on Hsp90.

Therefore, Silibinin caused the release of mature GR from Hsp90, so that more receptors are available in the cell to bind steroid with high affinity.

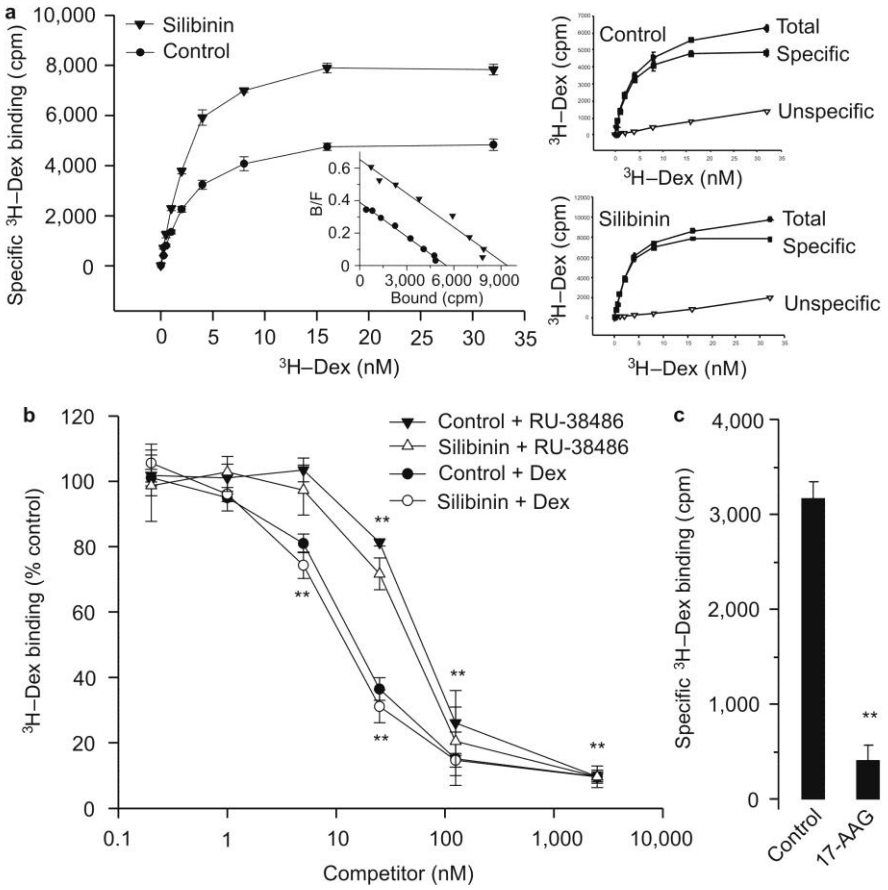

Figure 7: Binding of ^{3}H–Dexamethasone to GR in AtT–20 cells. (**a**) The effects of 30 μM Silibinin on the steroid binding activity of GR. Specific binding (left) is calculated from total minus unspecific (right) ^{3}H–Dexamethasone (^{3}H–Dex) binding. The Scatchard plot is derived from specific ^{3}H–Dex binding in cpm (B/F – bound/free). (**b**) Competition of binding of 5 nM ^{3}H–Dex by increasing concentrations of unlabelled Dexamethasone (Dex) or RU–38486 (**$P\leq0.01$; residual binding of ^{3}H–Dex alone vs. ^{3}H–Dex plus competitor). (**c**) Effects of 17–AAG on GR binding activity (**$P\leq0.01$). Data in **a**–**c** show means ± s.d. from representative experiments, each replicated twice.

THE C–TERMINAL HSP90 INHIBITORS SILIBININ AND NOVOBIOCIN POTENTIATE THE TRANSCRIPTIONAL ACTIVITY OF GR IN AtT–20 CELLS

In order to investigate the effects of Hsp90 inhibition on GR function, reporter gene assays were performed. AtT–20 cells were transiently transfected with the GR–responsive MMTV–Luc construct. Figure 9a shows the effects of 48–hour incubation with the indicated Hsp90 inhibitor. Dexamethasone was added for the last 18 h. The N–terminal Hsp90 inhibitor 17–

AAG dose dependently abolished GR activity, which is in line with the receptor degradation that was previously observed (see Figure 4). In sharp contrast, both Silibinin and Novobiocin potentiated the transcriptional activity elicited by Dexamethasone. This effect was dose–dependent and saturable, and neither inhibitor showed effects on MMTV–Luc activity in the absence of Dexamethasone (**Fig. 8a**).

The enhancing effect Silibinin and Novobiocin was also observed after pretreatment for 24 h and the addition of Dexamethasone for 6 h, except that the enhancement of GR activity by the compounds was less pronounced (**Fig. 8b**). Under these conditions, either of the two inhibitors increased GR activity to that elicited by a 10–fold higher concentration of Dexamethasone alone, whereas after 48 h treatment, the combination of C–terminal Hsp90 inhibitor plus Dexamethasone increased GR activity above that observed with a 10–fold higher concentration of Dexamethasone alone (see Fig. 8a). When AtT–20 cells were treated for 6 h and Dexamethasone was added for further 3 h, only a minimal enhancement of GR activity was observed for the highest concentration of Silibinin or 200 µM Novobiocin, while 300 µM Novobiocin showed a slight suppression of GR activity (**Fig. 8c**). For comparison, Silibinin and Novobiocin also showed weak effects of Hsp90 inhibition at short incubation times as measured by the stability of Cdc2 (see Fig. 4b).

To confirm that the effects of Silibinin are indeed mediated specifically by GR, the synthetic GR antagonist RU–38486 was used. As shown in Figure 8d, the induction of MMTV–Luc by Dexamethasone was abolished in the presence of RU–38486. The potentiation of GR activity in the presence of Silibinin plus Dexamethasone was equally abolished in the presence of the antagonist (**Fig. 8d**).

Furthermore, when AtT–20 cells were transfected with a construct containing the minimal TK–promoter with two GR response elements fused upstream (GRE_2–TK–Luc), Silibinin potentiated the stimulation induced by Dexamethasone. In contrast, when a reporter construct which lacks the GR response elements upstream of the TK–promoter was transfected (TK–Luc), neither Dexamethasone nor Dexamethasone plus Silibinin induced its activity (**Fig. 8e**).

Together, these results show that the effects of Silibinin do not cause an unspecific increase of transcription on the reporter genes. The inhibition of Hsp90 with Silibinin specifically potentiates the activity of GR elicited by its agonist.

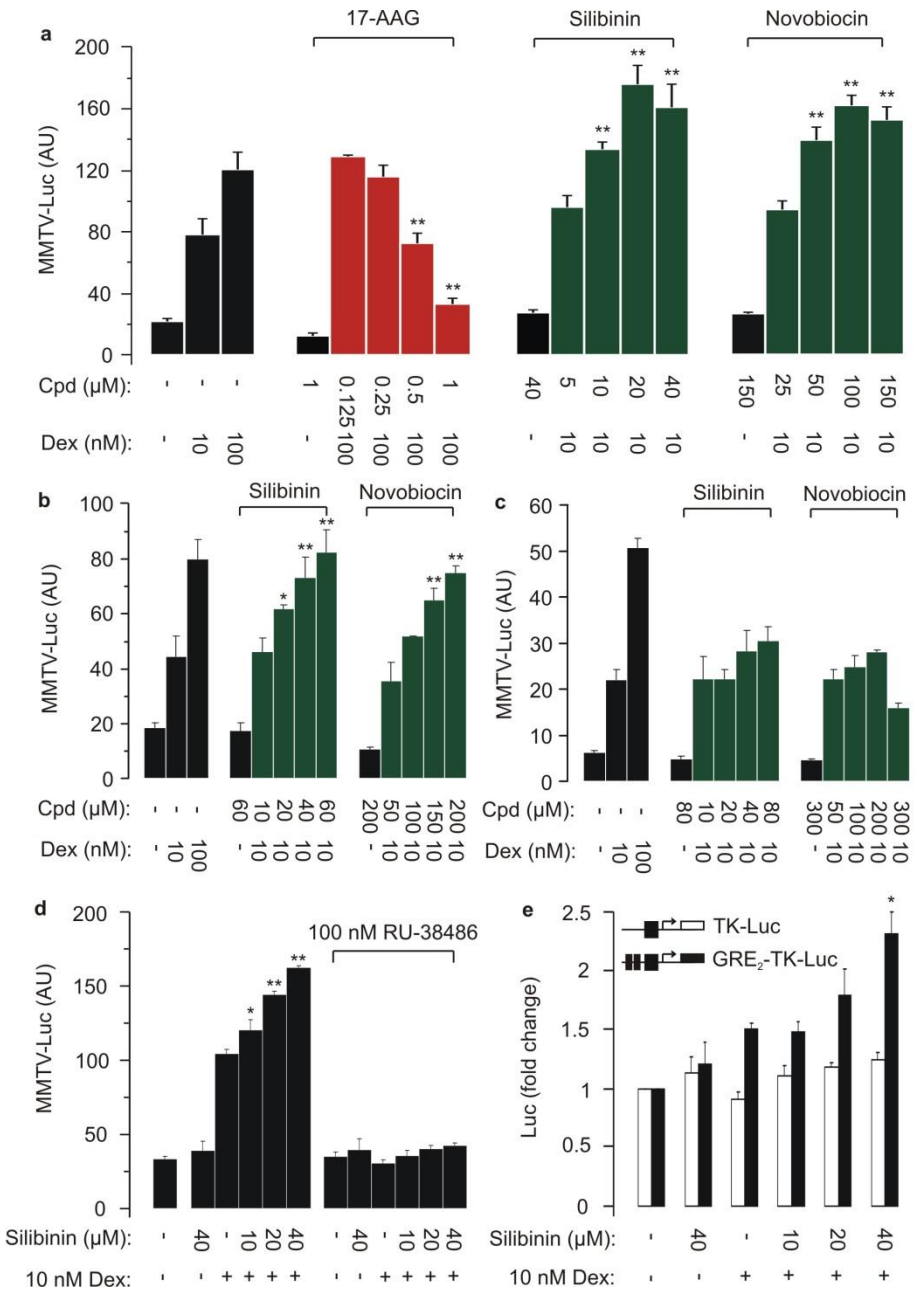

Figure 8: **Determination of GR activity in AtT–20 cells using reporter gene assays.** (**a**) Effects of 48–hour treatment with the indicated Hsp90 inhibitors on MMTV–Luc activity. Dex was added for the last 18 h; shown are means ± s.d. of one representative experiment (n = 4 total). (**b**) Effects of 24–hour treatment with the indicated Hsp90 inhibitors on MMTV–Luc activity. Dex was added for the last 6 h; shown are means ± s.d. (n = 2 experiments). (**c**) Effects of 6–hour treatment with the indicated Hsp90 inhibitors on MMTV–Luc activity. Dex was added for additional 3 h; shown are means ± s.d. (n = 2 experiments). (**d**) AtT–20 cells were treated as in **a**, with the exception that Dex and RU–38486 were added as indicated for the last 6 h; shown are means ± s.d. of one of two independent experiments. (**e**) AtT–20 cells were transiently transfected with TK–Luc or GRE_2–TK–Luc and treated for 48 h with Silibinin. Dex was added for the last 18 h; shown are means ± s.d. of fold change to control cells for two independent experiments (**P*≤0.05, ***P*≤0.01; compound plus Dex vs. Dex alone). AU stands for arbitrary units.

Altered Interaction Between a GR Mutant And Hsp90 Interferes With The Effects Of Silibinin

Due to the findings that C–terminal Hsp90 inhibitors promote the exit of GR from the chaperone in order to fulfil its role as a transcription factor in the presence of ligand, it was hypothesized here that a mutant of GR that displays altered interaction with Hsp90 would be influenced by Silibinin to a lesser extent than WT–GR.

To test this notion, the triple mutant P548A/T549A/V551A (AAA) of rat GR (Kaul et al., 2002) was overexpressed in AtT–20 cells. Silibinin showed a strong enhancing effect on WT–GR, while the mutant was minimally affected, and this effect was most obvious at half–maximal activity of each construct. As reported (Kaul et al., 2002), the mutant displayed a greatly reduced hormone responsiveness compared to WT (**Fig. 9**). The MMTV–Luc values gained for the activity of endogenous GR in a parallel control experiment (mock transfection) were below 15% of those measured for overexpressed WT–GR. Due to possible unspecific effects, higher concentrations of Dexamethasone were considered to not contribute additional valuable information.

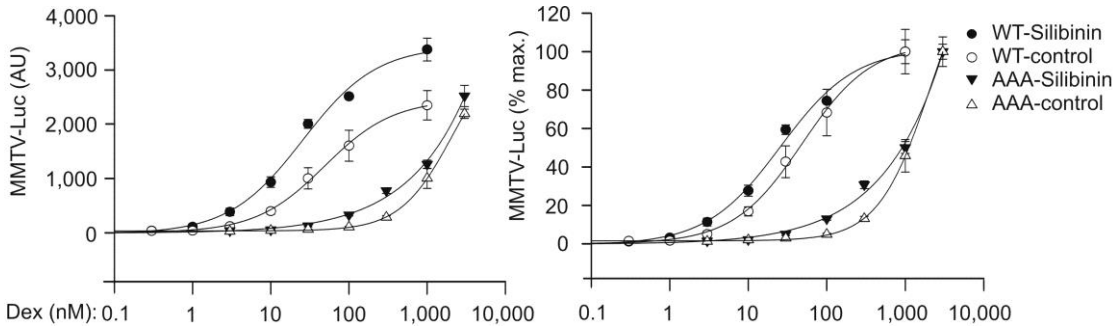

Figure 9: Effects of Silibinin on overexpressed WT and mutant GR. AtT–20 cells were treated for 48 h with Silibinin and Dex was added for the last 18 h. Maximal activity was set to the highest concentration of Dex applied (right); shown are means ± s.d. of one representative experiment (n = 2).

The Rapid Transport Of Agonist Bound GR Remains Functional In The Presence Of C–Terminal Hsp90 Inhibitors

The dependence of activated GR (holo–GR) on Hsp90 for the rapid and controlled nuclear transport was demonstrated (Czar et al., 1997). Since the reporter assays used here involved prolonged exposure to Dexamethasone (6 and 18 h), diffusion of GR into the nucleus might have taken place in the presence of the C–terminal Hsp90 inhibitors, which could still have resulted in the observed potentiation of GR activity. In order to investigate the effects of Hsp90 inhibitors on the rapid transport of holo–GR into the nucleus, immunocytochemistry was performed in AtT–20 cells. Figure 10 shows representative examples of nuclear

accumulation of GR after 15 min exposure to 10 nM Dexamethasone with or without pretreatment of the Hsp90 inhibitors. For AtT–20 cells which were pretreated for 15 min with 17–AAG, the staining of GR remained diffuse in the presence of Dexamethasone, reflecting inhibition of Hsp90 which hampers the transport of holo–GR into the nucleus as was reported for Geldanamycin (Czar et al., 1997). In contrast, after 48–hour treatment with Silibinin or Novobiocin, the nuclear accumulation of GR upon short exposure to Dexamethasone was comparable to that observed for control treated cells, while each inhibitor alone did not significantly influence the cellular localization of GR (**Fig. 10**). Thus, neither C–terminal inhibitor interferes with the molecular mechanism of Hsp90 which is necessary for the directed transport of activated GR into the nucleus.

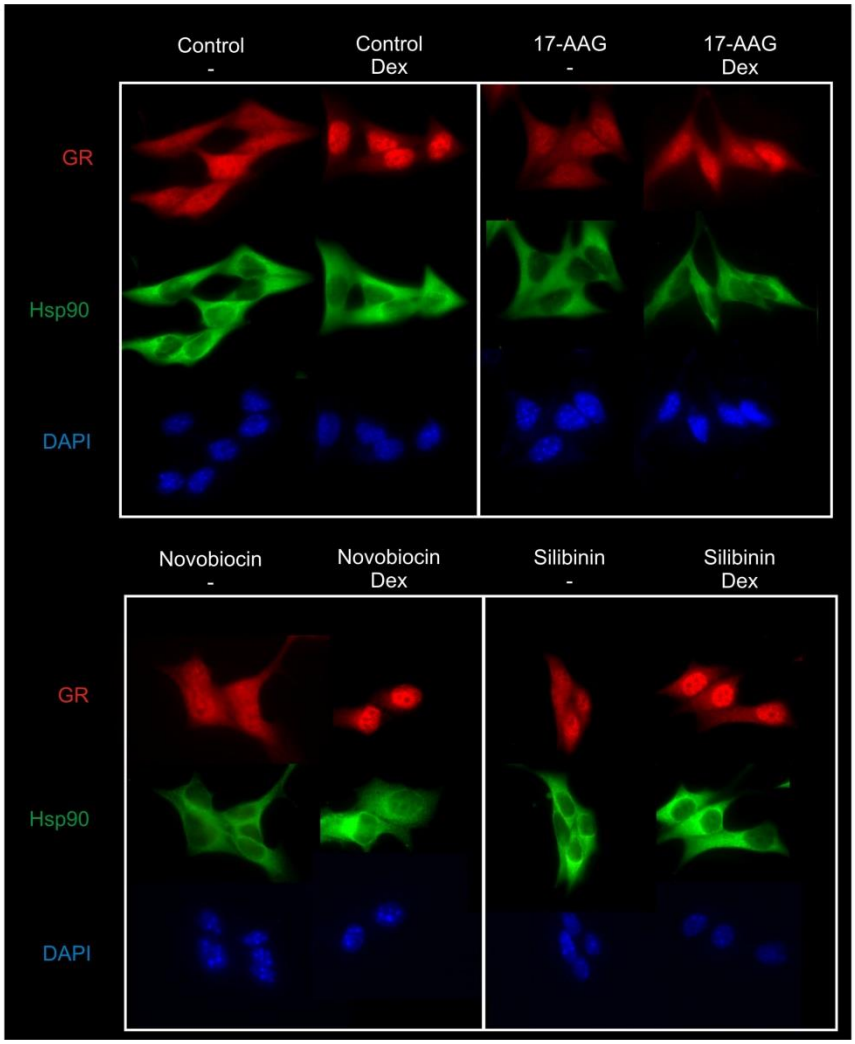

Figure 10: **Nuclear transport of GR.** AtT–20 cells were for 15 min with 10 µM 17–AAG, or for 48 h with 30 µM Silibinin or 100 µM Novobiocin. Dex was added for additional 15 min, cells were fixed and stained with DAPI. GR and Hsp90 were detected with specific antibodies and visualized using fluorescence microscopy.

SILIBININ ENHANCES THE SUPPRESSION OF ACTH MEDIATED BY GR IN AtT–20 CELLS

GR suppresses both basal and CRH–induced production of ACTH through *trans*–repression of the orphan receptor Nur77 in corticotroph cells as a monomer to recruit HDAC2, thus shutting down POMC gene expression (Philips et al., 1997b; Bilodeau et al., 2006). This molecular mechanism is different from *trans*–activation, in which binding of a GR dimer to GR response elements in the target genes recruits coactivators for the enhancement of gene expression. The fact that Silibinin potentiates GR *trans*–activation (MMTV–Luc and GRE_2–TK–Luc) led to the attractive notion that it might also enhance the suppression of endogenous ACTH in corticotroph adenoma cells. If this was the case, the partial Gc–resistance observed in patients with Cushing's disease could potentially be relieved by this small molecule.

First, this notion was investigated at the transcriptional level. AtT–20 cells were transiently transfected with the NurRE–Luc minimal construct or the POMC–Luc construct. These reporters contain three repeats of a Nur response element upstream of the POMC minimal promoter (Philips et al., 1997b), or the 770 bp of the rat POMC promoter and thus includes all the necessary sequences for the expression of POMC in pituitary cells *in vivo* (Liu et al., 1992), fused to luciferase, respectively. While Silibinin did not show effects on the basal activity of either reporter, the suppression of transcription elicited by low doses of Dexamethasone were potentiated for both constructs (**Fig. 11a**).

Next, it was investigated whether the effects of Silibinin on POMC activity resulted in a reduction of secretion of ACTH from AtT–20 cells. Therefore, AtT–20 cells in culture were treated for 48 h in medium containing 0.1% FCS to minimize effects of proliferative differences between the control and Silibinin treated cells. It was determined beforehand that – under these conditions – AtT–20 cells exhibit a normal cellular shape (see Fig. 10), that they hardly proliferate (WST–1), and that they do not show apoptotic DNA fragmentation (FACS) after 48 h (data not shown). In the experiments here, following hormone measurement, random wells were trypsinized and viable cells were determined by acridine orange/ethidium bromide staining followed by cell counting in a Neubauer counting chamber to confirm that the cell number did not significantly differ between the conditions (control: $5.1 \pm 0.5 \times 10^5$ cells ml^{-1} vs. 40 µM Silibinin: $4.8 \pm 0.6 \times 10^5$ cells ml^{-1}). Figure 11b shows that Silibinin did not influence basal ACTH production as measured by radioimmunoassay (RIA). However, the minimal suppression of ACTH by 1 nM Dexamethasone, which did not reach significance, was dose dependently enhanced by Silibinin. 10 nM Dexamethasone achieved a slight but significant suppression of ACTH, and this suppression was again potentiated by Silibinin (**Fig. 11b**).

For direct comparison, the effects of the N–terminal Hsp90 inhibitor 17–AAG on ACTH production in AtT–20 cells was investigated (**Fig. 11c**). There was a dose dependent reduction of basal ACTH secreted into the supernatant after 48 h of treatment. Notably, low concentrations of 17–AAG did not abolish suppression of ACTH mediated by GR. The highest dose of 17–AAG did not alter ACTH production when Dexamethasone was co–incubated, which likely reflects the observation that 17–AAG abolishes GR function at this concentration (see Fig. 8a).

In sum, it was confirmed that Silibinin results in the increased suppression of POMC transcription that is mediated by GR. As a consequence, the production of ACTH was significantly reduced when Dexamethasone was combined with Silibinin compared to Dexamethasone alone.

Silibinin Restores Glucocorticoid Sensitivity In Primary Cultures Of Human Corticotroph Adenomas

It was next sought to confirm that the molecular mechanism of Silibinin is also active in human corticotroph pituitary adenomas. To this end, primary cultures of biopsies from patients with Cushing's disease that had undergone transphenoidal surgery were produced. Figure 11d shows that 48 h treatment with 40 µM Silibinin did not have an effect on basal ACTH–production. However, the suppression elicited by 10 nM Dexamethasone during the last 24 h was variably, but significantly enhanced by Silibinin in 5 out of 6 specimens (**Fig. 11d**). Thus, Silibinin could potentially restore Gc–sensitivity in human corticotroph adenomas.

Silibinin Does Not Show Effects In Primary Cultures Of Rat Normal Pituitary Cells

Silibinin is reported to have an outstanding safety profile in animals and humans, with only mild adverse side effects even at high doses. It was investigated whether Silibinin treatment would influence normal corticotroph function, or permanently destroy normal pituitary viability: Apoptotic cells spill the storage vesicles filled with ACTH into the supernatant and result in strongly increased measurements. To this end, primary cultures from rat anterior pituitary were produced. RIA was performed to investigate the effects of Silibinin on ACTH production. Figure 11e shows that a concentration of Silibinin two–fold higher than that which elicited maximal effects in the corticotroph adenoma cells did not influence basal production or Dexamethasone suppression of ACTH (**Fig. 11e**).

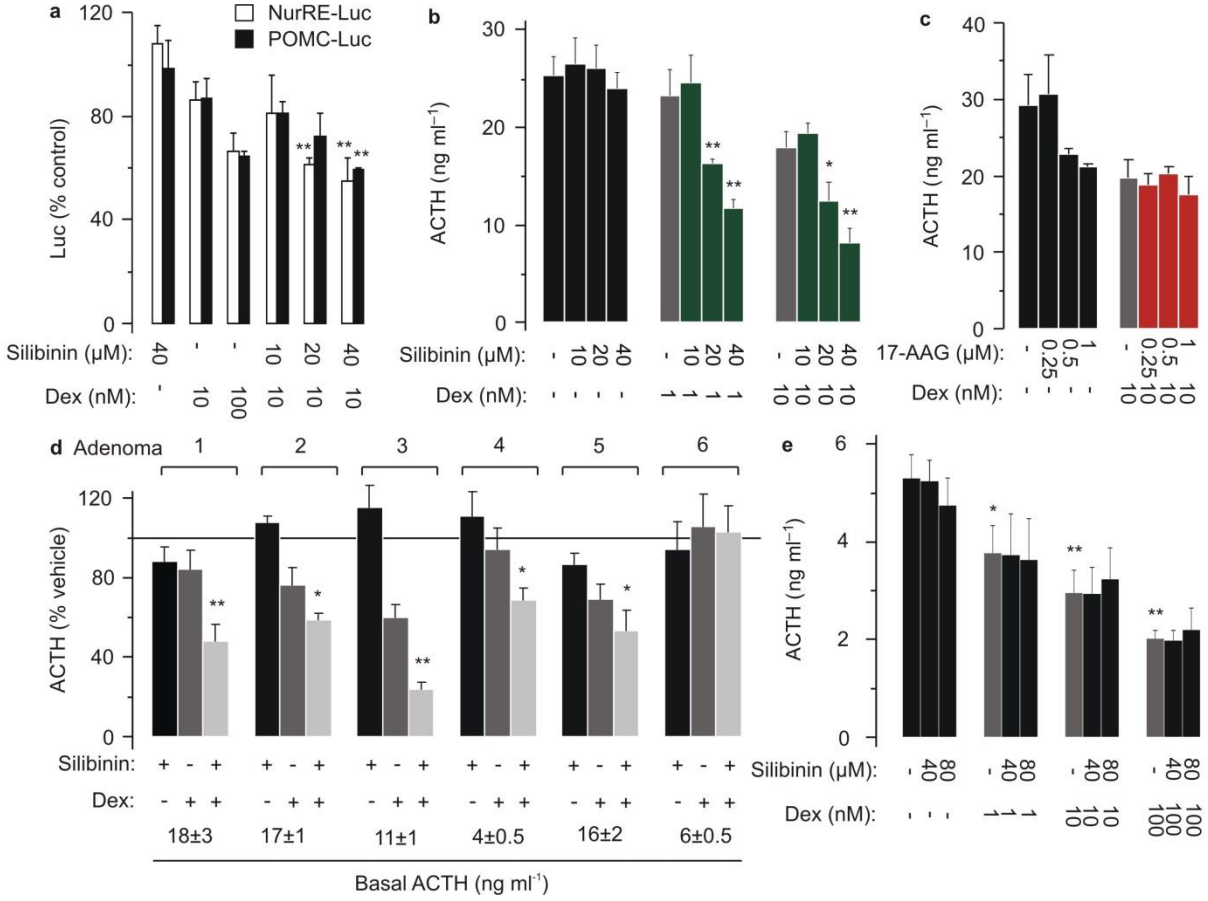

Figure 11: **Effects of Hsp90 inhibitors on the regulation of POMC and ACTH production.** (**a**) Treatment of AtT–20 cells for 48 h with Silibinin, with Dex added for the last 6 h. Normalized NurRE–Luc and POMC–Luc activity for control cells was set to 100%. (**b**) Silibinin and (**c**) 17–AAG differentially impact ACTH secretion in AtT–20 cells as measured by RIA. (**d**) Silibinin potentiates the suppression of ACTH mediated by GR in primary cultures of human corticotroph adenomas. Data in **a**–**d** show means ± s.d. Results in **a**–**c** are representative of three to five independent experiments (**P*≤0.05, ***P*≤0.01; compound plus Dex vs. Dex alone). (**e**) Silibinin does not affect GR activity in primary cultures of normal rat pituitary cells. Shown are means ± s.d. (**P*≤0.05, ***P*≤0.01; Dex vs. control).

SILIBININ SHOWS ANTITUMORIGENIC EFFECTS IN A MOUSE ALLOGRAFT MODEL FOR CUSHING'S DISEASE

AtT–20 cells produce tumors when injected subcutaneously into immunocompromised mice (Leung et al., 1982; Paez-Pereda et al., 2001). This model was used to determine the *in vivo* antitumorigenic efficacy of Silibinin. Daily administration of 300 mg Silibinin per kg bodyweight to male nude mice (n = 12 each group) implanted with AtT–20 allografts significantly reduced tumor growth as compared to the vehicle treated group after 28 days of treatment (**Fig. 12a**). Repeated measures ANOVA revealed that

Patients with Cushing's disease exhibit abnormal accumulation of central fat (moon face, buffalo hump) and tend to become obese (Feelders et al., 2012). Some of the phenotypical

symptoms observed in humans also display in hypercortisolemic mice which were implanted with AtT–20 cells (Leung et al., 1982). Symptoms which displayed in the mice were alleviated by Silibinin treatment as shown by the reduction in abnormal accumulation of fat deposits, and the less obese phenotype. Of note, the tumor of the Silibinin treated animal showed necrosis (**Fig. 12b**). The body weight of mice in the treatment group was lower compared to that of the vehicle group, but the difference did not reach significance.

The reduction in symptoms was paralleled by the enhanced suppression of plasma hormone levels by Silibinin. Measurement of ACTH and Corticosterone (Cort) levels showed an enhancing effect of Silibinin on the suppression of ACTH in the presence of high levels of circulating Corticosterone. In the treatment group, both ACTH and Corticosterone were reduced to approximately one third of the level observed in the vehicle treated group (**Fig. 12c**). Naïve animals without tumor (n = 3) and treatment served as control.

Taken together, these results reflect the *in vivo* efficacy of Silibinin. Furthermore, it was demonstrated that the molecular mechanism of Silibinin that was observed *in vitro* also is active in a mouse allograft model for Cushing's disease.

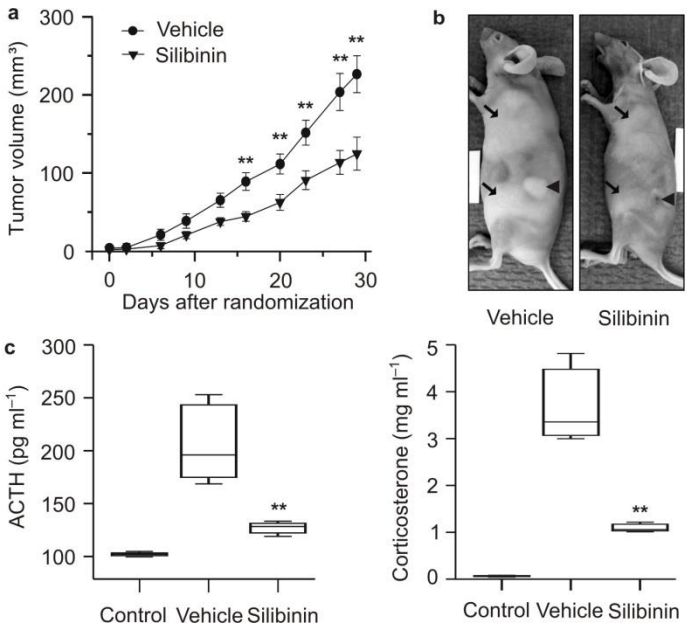

Figure 12: **Effects of Silibinin in an allograft model for Cushing's disease in mice.** (**a**) Seven days after AtT–20 cell injection, animals were randomized into vehicle (n = 12) and treatment group (n = 12). Suppression of the mean tumor volume ± s.e.m. in NMRI mice with AtT–20 tumor allografts by daily treatment of 300 mg Silibinin per kg body weight as compared to vehicle treatment (repeated measures ANOVA; ***P*<0,05). (**b**) Symptoms of representative animals in the vehicle and treatment group. Arrows denote abnormal fat pads which were reduced in the group that received Silibinin. Arrowheads point to the tumor. (**c**) Box plots of plasma ACTH and Corticosterone levels 24 h after the last treatment (Welch's *t* test; ***P*≤0.05; Silibinin vs. vehicle).

Note: Housing of the mice, feeding and drug administration, tumor injection, randomization, determination of tumor volume, and blood sampling were conducted by a third party, not by the author of the present work.

7 DISCUSSION

Patients with Cushing's disease suffer from chronic hypercortisolism which is caused by excessive secretion of ACTH from partially Gc–resistant corticotroph pituitary adenomas. To elucidate the molecular mechanism which underlies the partial Gc–resistance, genetic screens revealed that mutations of GR only explain the reduced hormone responsiveness in sporadic cases (Lamberts, 2002). Determination of the expression of the non–steroid binding isoform of GR could not explain the aberrant GR activity (Dahia et al., 1997). Brg–1 and HDAC2, cofactors of the transcriptional complex of GR necessary to suppress POMC were found to be abnormally expressed in less than half of the corticotroph adenomas investigated (Bilodeau et al., 2006). Hence, the molecular basis of the partial Gc–resistance of corticotroph adenomas is incompletely understood.

In the present work, it was hypothesized that abnormal expression or activity of the molecular chaperone Hsp90, the direct regulator of GR activity, causes the reduced sensitivity to Gcs in corticotroph adenoma cells. It was shown that human corticotroph adenomas strongly overexpress Hsp90α. Using distinct classes of Hsp90 inhibitors, strong antiproliferative effects and normalization of the response to Gcs were observed, indicating that Hsp90 overexpression is a pathogenic mechanism in corticotroph adenomas. These effects were explained by degradation of the oncogenic client protein Cdc2 with a concomitant cell cycle arrest, apoptotic DNA fragmentation, and the release of mature GR from Hsp90. The clinically safe Hsp90 inhibitor Silibinin enhanced the suppression of ACTH mediated by GR in AtT–20 cells and in primary cultures of human corticotroph adenomas. This explained the partial reversal of hormonal changes by treatment with the C–terminal Hsp90 inhibitor in the mouse allograft model for Cushing's disease, in addition to its antitumorigenic effects *in vivo*. In contrast, Silibinin did not affect the function of GR in rat normal pituitary cells.

THE SELECTION PROCESS OF THE HSP90 INHIBITORS

The benzoquinone ansamycin Geldanamycin was the first N–terminal Hsp90 inhibitor discovered (Whitesell et al., 1994). While it enabled researchers to gain insights into the function of Hsp90, it resulted in severe liver toxicity in a preclinical model (Supko et al., 1995). The toxicity of quinones is caused by their ability to produce reactive oxygen species due to redox cycling, as well as reacting with thiols to produce glutathione conjugates and adducts with cellular proteins (Guo et al., 2008). Due to its promising activity as anticancer agent *in vitro*, Geldanamycin served as the parent compound of 17–AAG, a first generation

Hsp90 inhibitor with greatly diminished intrinsic toxicity that proceeded to clinical phase II studies. After Geldanamycin showed severe cellular toxicity in AtT–20 cells even at concentrations when Hsp90 inhibition was not complete, it was decided to pursue further experiments with 17–AAG. It was a valuable process to observe the difference between a lead compound and a first generation derivative – at pronounced inhibition of Hsp90, the cells did not display signs of unspecific, toxic effects.

The aminocoumarin antibiotic Novobiocin is a C–terminal Hsp90 inhibitor. When cell cycle distribution was investigated in AtT–20 cells, Novobiocin caused an arrest at the G2/M phase at 24 h of treatment, whereas an increase in cells in subG1 phase was observed after 48–hour treatment. Silibinin, the second C–terminal Hsp90 inhibitor, arrested AtT–20 cells caused a G2/M arrest also at 48 h. This difference may be explained by unspecific, toxic effects of Novobiocin on AtT–20 cells. Indeed, a steep dose–response curve in the cell viability assay (WST–1 assay) suggests that this small molecule has intrinsic physicochemical properties which elicit cytotoxicity independent of its molecular target. This notion was corroborated when AtT–20 cells showed clear signs of toxicity under treatment with Novobiocin at concentrations higher than 500 µM. The majority of cells round up and detached from the culture plate within hours. Acridin orange/ethidium bromide staining revealed significant membrane disruption prior to morphological signs of controlled cell death. Furthermore Novobiocin showed an increase in apoptotic cells at the highest concentration used. In contrast, concentrations of 17–AAG which completely inhibit Hsp90 did not result in increased uptake of ethidium bromide, and the cells progressively displayed a distinct morphology which indicates apoptosis (i.e. normal morphology was preserved until membrane blebbing occurred, followed by cell death).

It is known that Novobiocin in high concentration binds to the N–terminal ATP binding pocket of Hsp90 in addition to the C–terminal domain (S+Âti et al., 2002), hence possibly acting like an N–terminal Hsp90 inhibitor. This notion may apply for the effects observed for two different assays in which 300 µM Novobiocin were used to treat AtT–20 cells. After 24 h, an increase in apoptotic cells was observed for the FACS analysis, an effect which is similar to that seen for 17–AAG. Also, 300 µM Novobiocin inhibited GR activity after 6 h, while lower concentrations increase GR activity.

The flavonoid Silibinin shows an outstanding safety profile in rodents as well as humans, even when high doses are administered (Saller et al., 2001; Hawke et al., 2010; Flaig et al., 2010). Depending on the formulation, Silibinin is readily adsorbed to reach plasma concentrations in the range of pharmacologic activity, ≥ 30µM in humans (Flaig et al., 2010).

Unfortunately, it is quickly conjugated in the liver for secretion (Wu et al., 2008). Silibinin has shown anticancerogenic effects in numerous types of cancer both *in vitro* and *in vivo.* Although not stated by the authors, seemingly pleiotropic effects of this small molecule led to the disruption of cancer pathways, mainly via targeted degradation of oncogenic kinases (Ramasamy and Agarwal, 2008). The evidence that the effects of Silibinin on cancer cells can be explained by the inhibition of Hsp90 is strong: Cell cycle arrest, the induction of apoptosis, or the inhibition of angiogenic processes were caused through the degradation of Cdk2, Cdk4, Cdc2 and Cdc25, WT– and mutant EGFR, Akt, Raf–1, IKK, MMP–2 and MMP–9, and HIF–1α (Dastpeyman et al., 2012; Rho et al., 2010; Tai et al., 2008; Ting et al., 2013). All of these proteins are prominent clients of Hsp90 and they display high sensitivity to its pharmacologic inhibition. Investigating two well–defined, endogenous Hsp90 clients in the cellular model AtT–20, where they fulfil physiologically relevant tasks, we found that Silibinin led to well defined effects which can be ascribed to the specific inhibition of Hsp90.

Additionally, Silibinin has been found to be effective in treating hepatic injury due to bile duct inflammation, cirrhosis, fatty liver, and mushroom poisoning (Pradhan and Girish, 2013). Consistent with the effects elicited by other flavonoids, Silibinin augments cellular antioxidant mechanisms involving glutathione and superoxide dismutase to scavenge free radicals, which could in part explain its effectiveness to prevent the induction of lipid peroxidation and cell death under certain pathologic conditions (Ligeret et al., 2008). Since the inhibition of Hsp90 using N–terminal inhibitors disrupts antioxidant mechanisms (De–Raedt et al., 2011), the diverging observations regarding this protective pathway may be attributed to distinct effects elicited by the C–terminal vs. N–terminal Hsp90 inhibitors, or by off–target effects of high concentrations of Silibinin.

It has been proposed that Silibinin is a selective ERβ agonist (Seidlov–Wuttke et al., 2003). However, additional reports showed contradicting results, stating that Silibinin either has estrogenic effects or anti–estrogenic effects in ovariectomized rats (Seidlov–Wuttke et al., 2003; Pliskova et al., 2005; Kummer et al., 2008). Longitudinal feeding studies over up to two years in rats and mice showed equal survival between groups, similar growth rates as well as body weight gain. Female mice did not display with estrous cycle abnormalities when fed with Silibinin, and a significant reduction of spontaneously formed mammary tumors was observed (Dunnick et al., 2011). Hence, it is unlikely that Silibinin elicits estrogenic effects. Considering that Silibinin may be efficacious in restoring the sensitivity of corticotroph adenomas to Gcs, this notion is of importance for the possibility of long term treatment of patients with Cushing's disease.

Diverging Effects Of The Hsp90 Inhibitors On Cell Cycle And Client Protein Stability

The anticancerogenic potential of Hsp90 inhibitors is based on the degradation of client proteins that drive oncogenesis and survival (Jhaveri et al., 2012). In order to be valuable as anticancer drugs, it was believed that all classes of Hsp90 inhibitors must act in the same way – the degradation of client proteins, which of course includes oncoproteins. In the corticotroph adenoma cell line AtT–20, the three distinct Hsp90 inhibitors 17–AAG, Silibinin and Novobiocin showed antiproliferative activity. However, diverging effects on cell cycle distribution were observed. While the N–terminal Hsp90 inhibitor 17–AAG caused an increase in hypodiploid cells, which is indicative of apoptotic DNA fragmentation, the C–terminal Hsp90 inhibitors Silibinin and Novobiocin arrested AtT–20 cells in the G2/M checkpoint of the cell cycle. Since all inhibitors led to the degradation of the Hsp90 client kinase Cdc2, an arrest at the G2/M checkpoint would have been expected also for 17–AAG. However, the rapid proteasomal degradation of most, if not all, proteins which require Hsp90 for function is observed in the presence of N–terminal Hsp90 inhibitors (Trepel et al., 2010). This may lead to the induction of apoptotic pathways that are dominant over cell cycle arrest, as was observed in AtT–20 cells. The molecular mechanism elicited by C–terminal Hsp90 differs from that of N–terminal Hsp90 inhibitors. Hsp90 bound to N–terminal inhibitors is isolated in cryptic, non–functional complexes with cochaperones that inefficiently binds to client proteins, which results in their degradation (An et al., 2000; Thulasiraman and Matts, 1996; Taipale et al., 2012). In contrast, the binding of C–terminal inhibitors results in a conformational change which reflects the client release conformation of Hsp90 with respect to kinases and cochaperones (Yun et al., 2004; Marcu et al., 2000a). These observations were confirmed in the present work, since both Silibinin and Novobiocin led to the release of mature GR from Hsp90 without influencing its cellular protein level, while 17–AAG caused the rapid loss of steroid binding activity of GR, followed by its degradation. Such functional differences between the two classes of Hsp90 inhibitors may cause the observed cell cycle arrest due to the predominant degradation of Cdc2 in the presence of Silibinin or Novobiocin in AtT–20 cells, which is only then followed by apoptosis.

The Dependence Of Cdc2 On Hsp90 In Corticotroph Adenoma Cells

Upon inhibition of Hsp90, AtT–20 cells displayed with cell cycle arrest at the G2/M phase due to the degradation of Cdc2, suggesting a particular role for the pathway of the cyclin–dependent kinase in corticotroph adenoma cells. This notion is corroborated by the fact that

pituitary adenoma formation is frequently observed in mice which lack the universal cyclin–dependent kinase (Cdk) inhibitor $p27^{Kip1}$ (Nakayama et al., 1996).

A recent work described the degree of dependence of client proteins on Hsp90, both with respect to their interaction, as well as their sensitivity to pharmacologic inhibition and subsequent degradation (Taipale et al., 2012). Using their assay, Cdc2 did not seem to be a client protein of Hsp90, which is in apparent contrast to the results obtained here and previous works (Nakai and Ishikawa, 2001; Garcia-Morales et al., 2007). Nakai *et. al* showed that when cellular Hsp90α levels were reduced, specific destabilization of Cdc2 was observed even under very mild heat stress (Nakai and Ishikawa, 2001). It can therefore be assumed that the overexpression of Hsp90α – as was observed in human corticotroph adenomas – leads to increased stability of this kinase, potentially driving oncogenic transformation. It is a well known fact that cancer cells with increased Hsp90 expression display with excessive stabilization of oncogenic client proteins, and thus, entire pathways (Whitesell and Lindquist, 2005). In turn, clients which are highly expressed, or increasingly unstable due to mutations, display enhanced dependence on Hsp90 (Xu et al., 2005; Patel et al., 2013). A cellular pathway driven by an oncogenic client protein is extremely susceptible to pharmacologic inhibition of Hsp90, unequivocally through the degradation of the client protein driving it. The degree to which a certain type of cancer is affected through the pharmacologic inhibition of Hsp90 is reflected by its dependence on this specific oncogenic pathway (Moulick et al., 2011; Patel et al., 2013; Isaacs et al., 2003). In corticotroph adenoma cells, Hsp90 inhibition results in the degradation of the oncogenic kinase Cdc2. Also the GR, which regulates ACTH production in normal corticotroph cells, and which is characterized by a partial resistance in corticotroph adenoma cells, is strongly affected by the inhibition of Hsp90.

C–Terminal Hsp90 Inhibitors Elicit a Novel Molecular Mechanism Which Leads To The Dissocation Of Mature GR From Hsp90

Observations made in which GR is artificially dissociated from Hsp90 in cellular lysate (increase in temperature, ionic strength, pH, dilution) have shown that GR cannot bind to Gcs under these conditions (Bresnick et al., 1989). Surprisingly, Silibinin and Novobiocin trigger the release of GR from the Hsp90 complex. Inside the living cell, this results in an increase of mature GR that keeps its high affinity for Dexamethasone, thus potentiating hormone responsiveness.

There are no reports on the action of Silibinin on GR signaling. However, our results with Novobiocin are in contrast to two earlier studies, in which higher concentrations inhibited the

maturation of GR in RRL and targeted it for degradation in Hela–cells (Kanelakis et al., 2002; Allan et al., 2006). This discrepancy is explained by the fact that Novobiocin in high concentrations binds to Hsp70 (Kanelakis et al., 2002) and to the N–terminal ATP binding pocket of Hsp90, in addition to the more sensitive C–terminal binding site (Söti et al., 2002) involved in the effects we report here.

Evidence that the effects of Silibinin on GR activity are due to the inhibition of Hsp90 is provided by the AAA–mutant of rat GR which was overexpressed in AtT–20 cells. The P548A/T549A/V551A mutations are located in a heptapeptide inside helix 1 at the very N–terminus of the GR–LBD (Bledsoe et al., 2002), and the presence of this motif is essential for the interaction of GR with Hsp90 (Xu et al., 1998). The mutant GR is activated to the same level as WT–GR in mammalian cells, however, it requires higher concentrations of dexamethasone to be activated (Kaul et al., 2002). Kaul *et al.* have shown that altered interaction of the AAA–mutant with Hsp90 leads to the observed reduction in steroid binding activity when this protein complex is in its dynamic state, i.e. within the cytoplasm of a living cell. If, in contrast, the AAA–mutant was matured by the five protein assembly system *in vitro*, which forces a stable GR::Hsp90 complex, it exhibits the same steroid binding activity as WT–GR (Kaul et al., 2002). Thus, the mutations do not abolish the ability of the GR–LBD to undergo structural changes necessary to acquire the high–affinity steroid binding conformation or its ability to act as a transcription factor, and constitute an appropriate model to investigate the effects of C–terminal Hsp90 inhibitors.

If the effects of Silibinin were not via inhibition of Hsp90, once activated, the activity of the AAA–mutant should be influenced similarly to that of WT–GR. While the AAA–mutant displayed with strongly reduced hormone responsiveness in AtT–20 cells, which is in line with previous observations (Kaul et al., 2002), Silibinin minimally affected its activity. This was expected if Silibinin influences ineffective interaction between the mutant and Hsp90. In contrast, WT–GR was strongly affected by Silibinin treatment, which is in line with the finding that Silibinin reliefs the inhibitory effect of Hsp90 on endogenous GR.

C–Terminal Hsp90 Inhibitors Potentiate GR Activity In Corticotroph Adenoma Cells

As was shown in a series of two control experiments, the potentiation of transcriptional activity through C–terminal Hsp90 inhibitors required GR which was bound to Dexamethasone: Addition of the synthetic GR antagonist RU–38486 abolished the effects of Dexamethasone alone and in combination with Silibinin. Furthermore, the activity of the

artificial GRE_2–TK–Luc construct was potentiated when Silibinin was incubated in addition to Dexamethasone, while neither Dexamethasone alone or in combination with Silibinin showed an effect on the TK–Luc construct which lacks the GR response elements.

The drug concentrations used to determine the effects on the transcriptional activity of GR were chosen according to the active inhibition of Hsp90 as determined by protein stability of Cdc2 and the absence of signs of cellular toxicity or a significant increase in apoptotic cells for all timepoints (FACS). The MMTV–Luc assay showed maximal effects for Silibinin and Novobiocin after 48 h of treatment. With respect to hormone responsiveness, the molecular mechanism elicited by this class of Hsp90 inhibitors is mainly due to its effects on apo–GR: Hsp90 has inhibitory activity on GR prior to Dexamethasone binding in corticotroph adenoma cells. The incubation with Silibinin or Novobiocin increasingly reliefs the inhibitory aspects of Hsp90 on GR over time. No effect was observed on MMTV–Luc activity when the compounds were incubated for 6 h, and Dexamethasone was added for additional 3 h. The addition of Dexamethasone leads to rapid conformational changes in GR, and the holo–GR::Hsp90 complex is distinct from the apo–GR::Hsp90 complex. With respect to the rapid, Hsp90–dependent translocation of holo–GR into the nucleus, C–terminal did not influence the interaction between GR and Hsp90 which follows binding of Dexamethasone. The effects of Hsp90 inhibition by these compounds after short incubation is minimal, as is reflected by the protein stability of Cdc2 at that time point. However, when AtT–20 cells were treated for 24 h with the C–terminal inhibitors, and Dexamethasone was subsequently added, their effects on MMTV–Luc activity were significant. For comparison, the degradation of Cdc2 was complete at that and at later timepoints. When Silibinin or Novobiocin was incubated for 30 h prior to the addition of Dexamethasone, a pronounced potentiation of GR activity was observed. This explains the similar effects of Silibinin and Novobiocin, because Dexamethasone was added at a timepoint when both inhibitors led to the G2/M arrest, which is specific for the inhibition of Hsp90 with this class of small molecules in AtT–20 cells.

The finding that C–terminal Hsp90 inhibitors potentiate the activity of GR through a mechanism that targets Hsp90 was corroborated by the fact that Silibinin also enhances the *trans*–repression of Nur77, and thus, POMC transcription. This consequently results in the reduction of ACTH in AtT–20 cells and in primary cultures of human corticotroph adenomas by Silibinin.

The mechanism of Hsp90 action on GR maturation in corticotroph adenoma cells is different from – and might act in addition to – the dissociation of transcriptional complexes through Hsp90 in other cell types (Kang et al., 1999; Freeman and Yamamoto, 2002), as well as other

mechanisms that contribute to glucocorticoid–resistance in corticotroph adenoma cells, such as the down–regulation of cofactors of GR or mutations in GR (Bilodeau et al., 2006; Kino et al., 2003). In the present work, while Silibinin enhanced the effects of Dexamethasone on ACTH production in the majority of primary cultures from human corticotroph adenomas, one culture out of six did not react on Dexamethasone, or on the combination of Dexamethasone plus Silibinin. It is possible that such mechanisms are responsible for the complete absence of GR activity, as was observed in this adenoma.

Functional Aspects Of Hsp90 Overexpression In Corticortoph Adenoma Cells

How can the inhibition of Hsp90 with C–terminal inhibitors target the kinase Cdc2 for degradation, while the GR is not affected at the cellular protein level, and released from Hsp90 in a mature state that binds Dexamethasone with high affinity? First, GR and kinases interact with distinct domains in Hsp90 during the catalytic cycle, and they require different cochaperones (Fang et al., 2006; Vaughan et al., 2006; Grad and Picard, 2007).

Furthermore it is possible that the overexpression of endogenous Hsp90 in corticotroph adenoma cells directs the composition of the Hsp90–cochaperone complexes into a direction which can efficiently fold and stabilize kinase clients – thereby driving tumorigenesis – but which may exert the observed inhibitory effects on the function of GR. Cochaperones ultimately regulate client recruitment as well as the catalytic cycle of Hsp90 (Röhl et al., 2013). Dynamic changes in the composition of Hsp90–cochaperone complexes due to the overexpression of certain components of the chaperone machinery result in impaired activity of nuclear hormone receptors in particular (Davies et al., 2005; Laenger et al., 2009; Schuelke et al., 2010). Intriguingly, certain post–translational modifications enhance the ATPase activity of Hsp90, which results in disturbed capacity to chaperone client proteins, including GR (Mollapour et al., 2014). Similar observations were made when the catalytic cycle of Hsp90 was accelerated through overexpression of Aha1 (Wang et al., 2006).

Hence, if the stoichiometry of Hsp90 and cochaperones can influence the conformational flexibility which constitutes the catalytic cycle of Hsp90, overexpression of Hsp90 may influence client protein fate to a similar extent than mutations in Hsp90. All mutations identified in Hsp90 are detrimental with respect to the chaperoning of client proteins. Isolated were point mutants of Hsp90 which cannot bind ATP (Panaretou et al., 1998), which bind ATP but show weak hydrolase activity (Xu et al., 2012), mutants that prevent post–translational modifications (Retzlaff et al., 2009; Mollapour et al., 2010; Mollapour et al., 2014), or those selected for reduced ability to sustain yeast cell viability as well as client

protein function under ambient or elevated temperature (Bohen and Yamamoto, 1993; Nathan and Lindquist, 1995; Hawle et al., 2006). It can be concluded that mutations in Hsp90 either interfere with the necessary conformational flexibility of the chaperone, that they distort motifs which may be required for client and/or cochaperone binding, or that the ATPase activity is abolished. While mutations constitute stable changes in a protein, the overexpression of Hsp90 can be targeted by small molecule compounds. In corticotroph adenoma cells, C–terminal Hsp90 inhibitors influence the function or the conformation of Hsp90. This results in the relief of inhibitory effects of Hsp90 on the maturation of GR without influencing its transport to the nucleus, while Cdc2 is targeted for degradation. On the other hand, Silibinin does not alter GR activity in normal pituitary cells. The tumor selectivity of Hsp90 inhibitors depends on hyperactive Hsp90α complexes in other malignancies (Kamal et al., 2003; Moulick et al., 2011). The absence of effects of Silibinin in normal pituitary cells may be explained by the low expression levels of Hsp90α.

Interpretation Of The AtT–20 Allograft Mouse Model

The allograft mouse model study was designed and conducted based on previous experience and reports with respect to the expected effect size, unilateral allograft injection, the start of treatment prior to the detectable formation of tumors (Leung et al., 1982; Paez-Pereda et al., 2001), and drug dosage (Vogel et al., 1975). After 29 days of treatment, animals in the vehicle group uniformly presented with severe symptoms caused by the hypercortisolism, and it was decided to sacrifice the mice in order to be able to collect blood samples for hormone analyses from all animals in both groups 24 h after the last treatment. The reduction in allograft tumor volume as well as plasma hormone levels was significant in the animals which received Silibinin, which coincided with a relief of Cushingoid symptoms. By definition, a partial biochemical control of plasma or – more commonly – urinary free cortisol levels in patients with Cushing's disease is defined by a ≥ 50% reduction to baseline (Colao et al., 2012). In the present study, Silibinin achieved this goal when the treatment group was compared to the vehicle group. Nevertheless, the hormonal levels in the Silibinin group were still strongly elevated compared to those measured in naïve control animals which received no treatment. The procedure of the oral drug administration could, in part, have contributed to the increased stress hormone levels in the study animals. Furthermore, the rapid clearance from the organism, or the tumors, may have hampered the efficacy of Silibinin in the allograft mouse model. In a human phase II clinical trial for patients with prostate cancer, Silibinin did not reach significant concentrations inside the tumors, which explained the absence of its effects

(Flaig et al., 2010). While the effects of Silibinin were strong in the mouse allograft model presented here, more favorable formulations of Silibinin that result in high plasma levels may achieve a biochemical control of patients with Cushing's disease in the future.

CONCLUSION

In the present work, a pathogenic mechanism is described in which the strong overexpression of Hsp90α in corticotroph adenoma cells exerts inhibitory effects on GR for the binding of Gcs (**Fig. IV – 1**), and that this inhibitory mechanism is reversible using small molecules which target the C–terminal client binding domain of Hsp90 (**Fig. IV – 2**). The novel biochemical activity elicited by Silibinin and Novobiocin involve the release of mature GR from Hsp90, such that more receptor is available in the cell to bind Gcs with high affinity. Since C–terminal Hsp90 inhibitors the binding of Gcs to GR, both *trans*–activation and *trans*–repression are enhanced. Through the increased suppression of ACTH mediated by GR, Silibinin reverses the partial Gc–resistance in human corticotroph adenomas. In normal corticotroph cells, GR senses physiological Gc–levels and Hsp90α shows low expression. In this cell type, the function of GR remains unaffected by the inhibitor (**Fig. IV – 3**). All together, it is shown that the clinically safe Hsp90 inhibitor Silibinin could be used to treat patients with Cushing's disease.

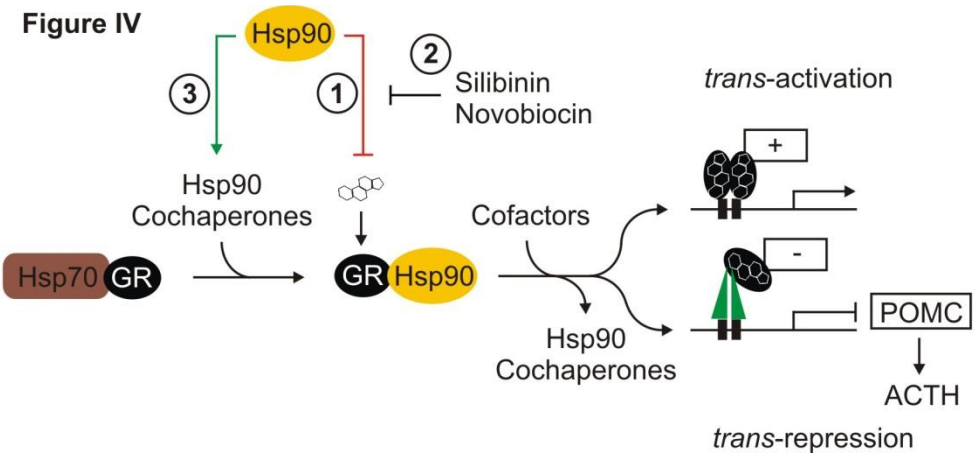

Figure IV: Summary of the effects of Hsp90 overexpression on GR activity in corticotroph adenomas and the potential restoration of Gc–sensitivity with C–terminal Hsp90 inhibitors.

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Steff, ich möchte Dir dafür danken, dass Du eine so gute Mutter bist. Und ohne Euch, meine liebe Familie, wäre ich nicht wer und wo ich bin – ich danke Euch.

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe nicht anderweitig versucht, eine Dissertation oder Teile einer Dissertation einzureichen beziehungsweise einer Prüfungskommission vorzulegen, noch mich einer Doktorprüfung zu unterziehen.
Da das Max–Planck–Institut für Psychiatrie nicht über die Einrichtungen zur Haltung von Mäusen mit geschwächtem Immunsystem verfügt, wurden folgende Arbeiten nicht von mir, sondern von einer externen Firma, durchgeführt: Die Haltung der Tiere, die Injektion der Tumorzellen, die Randomisierung, die Verabreichung von Silibinin oder Vehikel, die Messung der Tumorvolumina, und die Entnahme von Blut.

München, im November 2014

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Mathias Riebold