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Silibinin, an HSP90 Inhibitor, on Human ACTH-Secreting Adenomas

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Silibinin · Heat shock protein 90 · Cushing's disease · Glucocorticoid feedback

Abstract
Introduction: The glucocorticoid receptor is pivotal to control corticotrophin (ACTH) secretion, and its function is closely linked to the heat shock protein 90 (HSP90) chaperone complex. Impaired sensitivity to glucocorticoid feedback is a hallmark of human corticotroph adenomas, i.e., Cushing's disease, a disorder with few medical treatment options. Silibinin, a HSP90 inhibitor, has been studied in tumoral corticotroph cells and its use proposed in Cushing's disease. Aim of the present study was to further investigate the effect of silibinin on human corticotroph adenomas in vitro. ***Methods:*** Seven human ACTH-secreting pituitary adenomas were established in culture and treated with 10–50 μM silibinin with/without dexamethasone for up to 72 h. ACTH medium levels were measured, and *POMC* and glucocorticoid receptor, i.e., *NR3C1*, gene expression assessed. ***Results:*** Silibinin reduced spontaneous ACTH secretion and restored sensitivity to steroid negative feedback to a different extent in individual adenomas. *POMC* expression was decreased in both control and dexamethasone-treated wells in specimens sensitive to silibinin. Interestingly, silibinin reduced constitutive *NR3C1* expression and reversed the dexamethasone-induced inhibition. ***Conclusions:*** Our findings indicate that silibinin can inhibit ACTH synthesis and secretion in individual human corticotroph adenomas and directly affects *NR3C1* gene expression. These results reveal promising effects of this HSP90 inhibitor on human corticotroph adenomas and support an innovative target treatment for patients with Cushing's disease.

Introduction

Cushing's disease is a rare, highly morbid endocrine disorder with few medical options. Indeed, patients who fail or relapse after surgery are usually treated with a variety of drugs as none assures optimal disease control. Drugs may be aimed at the pituitary, i.e., control of ACTH secretion, or act downstream, on the adrenal, to reduce cortisol secretion. The former represents the more rational approach, as the tumoral corticotroph is the cause of excess cortisol secretion in Cushing's disease. However, success rates of approved as well as off-label treatments, e.g., pasireotide, cabergoline, retinoic acid, reach 30% at most and escape from disease control is not uncommon. Adrenal-directed drugs achieve

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control of hypercortisolism in over half of patients but may lead to adverse events. On balance, no long-term safe and efficacious medical therapy is currently available to patients with Cushing's disease [1].

Given the above, research into the pathophysiology of tumoral corticotrophs has attempted to better elucidate mechanisms underlying altered ACTH synthesis and secretion, in particular impaired sensitivity to negative glucocorticoid feedback [2]. In our recent study on over 70 corticotroph adenomas, a reduction in ACTH secretion and *POMC* expression was observed in less than half, the remainder unaffected by dexamethasone or even presenting paradoxical increases [3, 4]. The effect of glucocorticoids on corticotrophs is known to involve chaperone and co-chaperones – primarily responsible for transfer of the liganded receptor dimer complex to the nucleus – as well as transcription factors deputized to control of DNA binding and transcription regulation [5]. Both liganded and unliganded glucocorticoid receptors shuttle between cytoplasm and nucleus [6], and the equilibrium between corepressors and coactivators has been shown to modulate activity of the glucocorticoid receptor complex [7].

Heat shock protein 90 (HSP90), an ubiquitous, stress-induced, cytosolic protein, was among the first chaperones to be associated with the glucocorticoid receptor [8]. In the context of corticotroph adenomas, recent studies showed that the HSP90 protein is present in ACTH-secreting adenomas with both HSP90 isoforms, i.e., alpha and beta, abundant compared to normal pituitary and non-secreting adenomas [9]; indeed, overexpression of HSP90 is believed to underlie partial resistance to glucocorticoid feedback [9], one of the hallmarks of ACTH-secreting pituitary adenomas [10].

Given this evidence, it stands to reason that HSP90 inhibitors may modulate ACTH secretion by tumoral corticotrophs. Indeed, both natural and synthetic compounds have been tested in experimental models, i.e., silibinin, a milk thistle extract, among the former, and 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) and CCT018159, among the latter [9, 11, 12]. Studies on the murine corticotroph cell line AtT-20 indicated that HSP90 inhibitors inhibit *POMC* transcription and ACTH secretion in vitro and blunt tumor growth and fat deposition in mice carrying AtT-20 allografts [9, 11, 12]. Silibinin and 17-AAG were also tested on human corticotroph adenomas and revealed enhanced suppression during dexamethasone incubation at 24 h [9] and reduced spontaneous ACTH synthesis and secretion – possibly due to decreased cell viability – after 48 h [12]. Aim of the current study was to expand current knowledge on the effect of silibinin on ACTH synthesis and secretion as well as expression of the glucocorticoid receptor itself in human corticotroph adenomas in order to pave the way to target therapy for Cushing's disease.

Materials and Methods

Pituitary Adenoma Primary Cultures

Seven ACTH-secreting adenomas were collected in patients diagnosed with Cushing's disease (4 males, 3 females, age range 29–50 years, 3 macroadenomas) and specimens cultured according to our protocol [3, 4, 13]. In detail, adenomas were dispersed by enzymatic digestion and plated at 20×10^3 to 300×10^3 per well, according to specimen abundance. Cells were attached in DMEM, 10% fetal calf serum and antibiotics for 3–5 days, then washed with DMEM and 0.1% BSA prior to challenge with 10–50 μM silibinin (CAS registry # 22888-70.6, reference standard analysis: RST Code 0291001, IDN Code 1014, 99.11% purity), a gift by Istituto Biochimico Italiano-Giovanni Lorenzini (Aprilia, Italy) alone or with 10 nM dexamethasone. Silibinin stock was resuspended in distilled water, diluted and aliquoted; a single aliquot was used for each experiment. Control wells were incubated with DMEM + 0.1% BSA alone and each treatment was performed in triplicate. All reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA. After 4-, 48-, and 72-h incubation, medium was collected for ACTH measurement and cell RNA extracted (TRIzol reagent, Invitrogen, Milan, Italy).

ACTH Assay

ACTH was measured by immunoradiometric assay (DiaSorin, Saluggia, Italy) according to manufacturer's instructions; sensitivity and intra-assay coefficient of variation are 1.2 pg/mL and 5.9%, respectively. All samples from a given specimen were measured in the same run. Only pituitary cultures secreting at least 10 pg/10^5 cell ACTH in basal conditions were included in the study, in order to ensure the presence of tumoral corticotrophs [3, 14]. Indeed, normal corticotrophs are silenced by long-standing hypercortisolism in patients with Cushing's disease as over 1 year is usually required for recovery of normal corticotrophs [15] and reappearance of the ACTH response to CRH [16]. Within specimen variability of spontaneous ACTH secretion, calculated as the coefficient of variation (i.e., SD/mean), averaged 7%; a difference of at least 20% from control ACTH secretion, i.e., >two-fold greater than the variability of spontaneous secretion, was taken to indicate a response [17].

Quantitative Real-Time PCR

RNA (100 ng) was reverse-transcribed by SuperScript VILO cDNA Synthesis Kit (Invitrogen) and quantitative real-time PCR performed on a 7900 HT sequence detection system (Applied Biosystem, Foster City, CA, USA), using Platinum Quantitative PCR SuperMix-UDG with premixed ROX. TaqMan assay (Applied Biosystem,) was used for detection of *POMC* (probe Hs00174947_m1) and *NR3C1,* i.e., glucocorticoid receptor (probe Hs00230813_m1) with *RPLP0* as housekeeping gene (probe Hs99999902_m1) [4]. The melting curve of PCR products allowed separation of genuine products from non-specific products and primer dimers. Basal expression data ($2^{-\Delta Ct}$) were calculated and normalized to *RPLP0*; expression after treatment was analyzed as $2^{-\Delta\Delta Ct}$ and expressed as fold change versus control [4].

USP8 *Sequencing*

Sequencing from primary culture specimens was performed as described in [18]. In brief, 100 ng RNA was reverse-transcribed with primers: 5'CTTGACCCAATCACTGGAAC 3' (forward) and 5'TTACTGTTGGCTTCCTCTTCTC 3' (reverse) for amplification of *USP8* exon 14, the most frequent site of mutations reported so far [19]. Touch-down PCR was performed using GO TAQ DNA polymerase (Promega, Madison, WI) at 64°C–57°C annealing. PCR products were purified by ExoProStar Illustra enzyme (Ge Healthcare, Chicago, IL, USA) and Sanger sequencing performed using the ABI PRISM BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) on ABI PRISM 3500 analyzer [20]. All chromatographs yielded informative sequencing data.

Statistical Analysis

Friedman's non-parametric test followed by least significant difference inequality according to Conover [21] was used to assess changes in ACTH concentrations across silibinin treatments with and without dexamethasone in individual specimens. Changes in *POMC* and *NR3C1* during silibinin or dexamethasone alone were evaluated by Wilcoxon rank-sum test, as were the effects of dexamethasone on ACTH secretion. Significance was accepted for $p < 0.05$.

Results

Silibinin reduced spontaneous ACTH secretion in most adenomas as shown in Figure 1 (details of statistical analyses are given in online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000529710). In detail, significant decrease could be observed at 4 and 48 h in 5 adenomas; in 4 specimens, silibinin significantly decreased ACTH up to 72 h, shown in Figure 1. In specimens sensitive to silibinin, i.e., decrease by at least 20% of spontaneous secretion [17], comparable responses could be observed at 10 μM, 20 μM, and 50 μM (raw data https://doi.org/10.13130/RD_UNIMI/QBRJNN).

Evaluation of sensitivity to glucocorticoid negative feedback revealed marked inhibition of ACTH secretion during incubation with dexamethasone in 2 adenomas (#1, #4), while ACTH concentrations were unaffected by dexamethasone in 2 (#3, #6) and three specimens exhibited a paradoxical increase (#2, #5, #7) in ACTH, shown in Figure 2, as has been shown to occur across tumoral specimens in vitro [3]. Interestingly, silibinin could enhance as well as restore sensitivity to dexamethasone (details of statistical analyses are given in online suppl. Table 1). In fact, silibinin further decreased ACTH concentrations in specimens #1 and #4 and restored sensitivity to negative feedback in specimens #2 and #3, the former exhibiting a paradoxical increase, the latter unaffected by dexamethasone alone, shown in Figure 2. The sensitizing effect of silibinin lasted up to 72 h in all 4 adenomas. No effect of silibinin on ACTH concentrations during dexamethasone incubation was observed in specimens #5, #6, and #7, as shown in Figure 2. As with spontaneous ACTH secretion, responsivity to silibinin was comparable within the tested concentration range.

Silibinin significantly reduced *POMC* mRNA in 5 out of 7 adenomatous specimens (details of statistical analyses are given in online suppl. Table 1): a decrease by 30–60% was observed in adenomas presenting decreased ACTH secretion during silibinin incubation as shown in Figure 3a. In wells co-incubated with 10 μM silibinin and dexamethasone, a significant reduction in *POMC* mRNA compared to the effect of dexamethasone was observed in 4 specimens in which a decrease in ACTH levels was observed, as shown in Figure 3b. Similar results were observed with 20 μM and 50 μM.

Silibinin induced a marked decrease in *NR3C1* in 4 specimens (#1, #2, #3, and #4; shown in Fig. 4a; details of statistical analyses are given in online suppl. Table 1). Dexamethasone itself reduced expression of its receptor in most ACTH-secreting adenomas, as we have previously shown [4]. Silibinin reversed dexamethasone-induced *NR3C1* suppression with two- and three-fold increases in glucocorticoid receptor gene expression in specimens responsive to silibinin, as shown in Figure 4b.

USP8 sequencing revealed the p.S718P variant in two adenomas (#3 and #4), the remainder presenting the wildtype sequence. Both *USP8* variant specimens were responsive to silibinin, whereas wildtype adenomas exhibited both responsive and non-responsive patterns. No difference as regards sensitivity to silibinin was observed between adenomas from male (#1, #2, #5, and #7) and female (#3, #4, and #6) patients, in keeping with comparable responsivity to other corticotroph modulators, e.g., corticotrophin-releasing hormone, steroids, in vitro [22].

Discussion

Interest in the role of silibinin in Cushing's disease arose from the observation that this hepatoprotective agent interacts with HSP90 in tumoral corticotroph cells [9]. HSP90 is essential for proper folding of the glucocorticoid receptor, steroid binding, and movement of the ligand-bound HSP90-glucocorticoid receptor complex across the nuclear membrane [23–25]. Within the nucleus, HSP90 dissociates from the glucocorticoid receptor complex allowing binding to glucocorticoid receptor elements and initiation of gene transcription and, ultimately, glucocorticoid receptor recycling. At the same time, continued binding of

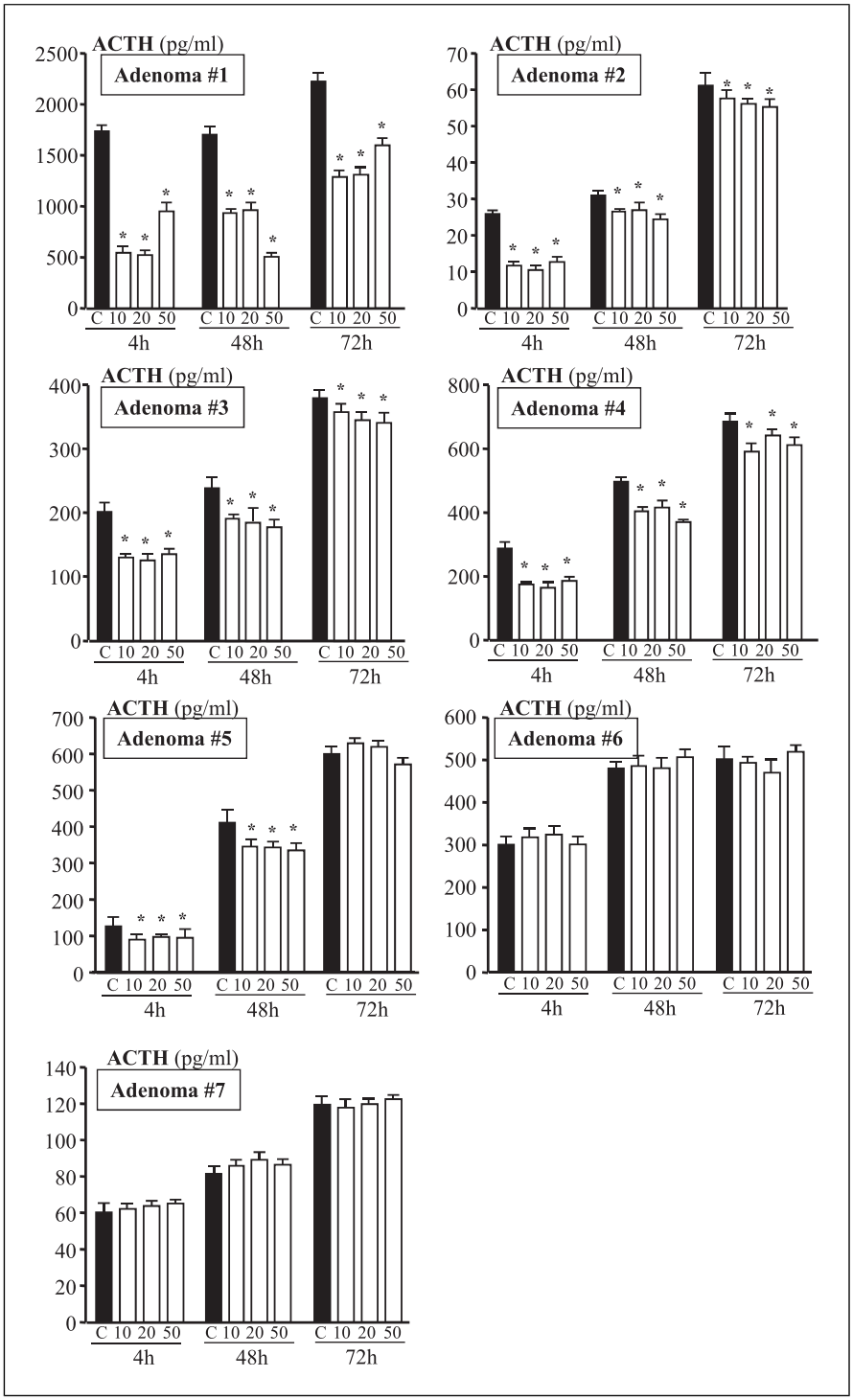

Fig. 1. ACTH concentrations in ACTH-secreting pituitary adenoma specimens during incubation with silibinin. Black bars represent control wells (C), white bars wells incubated with silibinin at 10, 20, and 50 μM. Bars represent mean ± SEM of triplicate wells; *indicates $p < 0.05$ silibinin versus control wells.

HSP90 to the glucocorticoid receptor protein is believed to hamper the formation of transcriptional complexes and inhibit transcriptional activity [26, 27]. Thus, the HSP90 machinery modulates glucocorticoid-induced transcription at several levels, and a dual, both positive and negative actions of HSP90 on glucocorticoid receptor-induced transcription, has been postulated to explain reduced signal transduction with low HSP90 levels [25] and attenuated receptor response with high HSP90 levels [26].

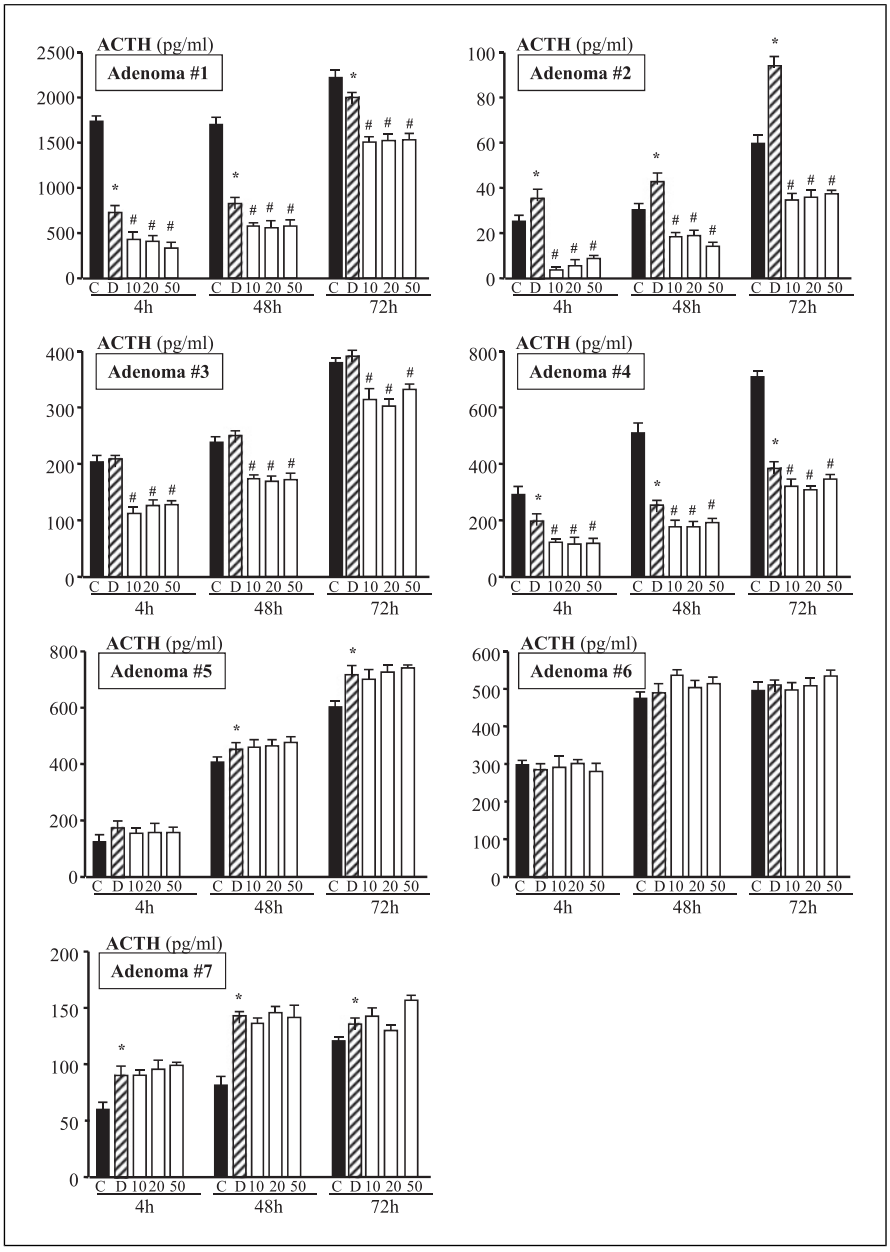

Fig. 2. ACTH concentrations in ACTH-secreting pituitary adenoma specimens during incubation with dexamethasone and silibinin. Black bars represent control wells (C), shaded bars indicate effect of 10 nM dexamethasone alone (D), and white bars indicate wells incubated with dexamethasone and 10, 20, and 50 μM silibinin. Bars represent mean ± SEM of triplicate wells; *indicates $p < 0.05$ dexamethasone versus control wells; # indicates $p < 0.05$ silibinin+ dexamethasone versus dexamethasone-treated wells.

A ground-breaking study showed that HSP90 is overexpressed in human corticotroph adenomas and extensively investigated HSP90 inhibitors in the murine tumoral corticotroph cell line [9]. These authors also assessed the effect of silibinin, a natural HSP90 inhibitor, on ACTH secretion in corticotroph adenomas. The aim of our study was to further investigate the role of silibinin in human tumoral corticotrophs, including time and dose-dependent responses as well as modulation of gene expression, in order to strengthen its role as a target therapy for Cushing's disease.

First, we can report that silibinin reduces spontaneous ACTH secretion in a considerable proportion of human corticotroph adenomas, albeit was variable in extent and duration, in keeping with the inherent diversity among ACTH-secreting adenomas [3, 20]. The previous study on human corticotroph adenomas failed to observe a clear reduction in baseline ACTH secretion after 24-h incubation with 30 μM silibinin [9]. In our hands, the effect was most evident after 4-h incubation and lessened somewhat over 48 and 72 h. Of note, this trend over time argues against an effect on cell viability, which has been

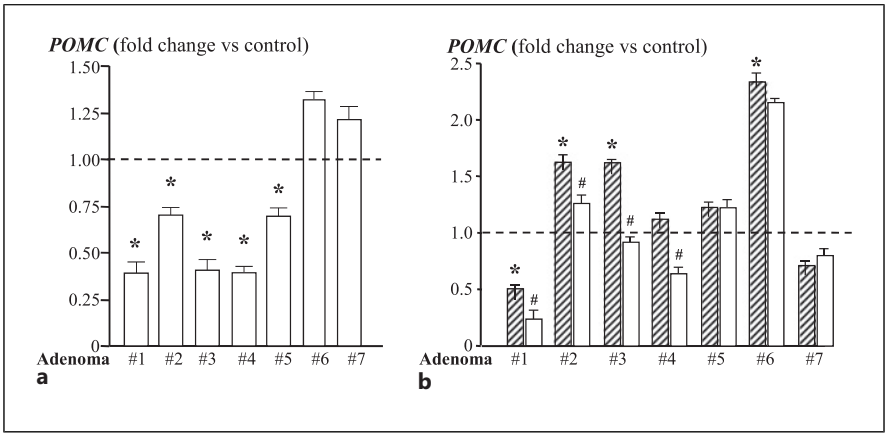

Fig. 3. *POMC* quantification in ACTH-secreting pituitary adenoma specimens after 72 h incubation with 10 μM silibinin (**a**) and silibinin + dexamethasone ((**b**) shaded bars: 10 nM dexamethasone; white bars: dexamethasone and 10 μM silibinin). Dashed line indicates *POMC* levels in control wells. Bars represent mean ± SEM of triplicate wells; *indicates $p < 0.05$ versus control wells; # indicates $p < 0.05$ silibinin + dexamethasone versus dexamethasone-treated wells.

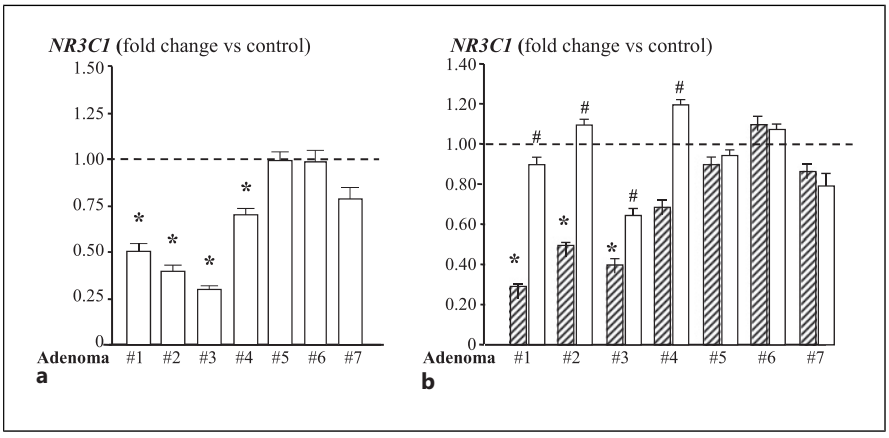

Fig. 4. *NR3C1* quantification in ACTH-secreting pituitary adenoma specimens after 72 h incubation with 10 μM silibinin (**a**) and silibinin + dexamethasone ((**b**) shaded bars: 10 nM dexamethasone; white bars: dexamethasone and 10 μM silibinin). Dashed line indicates *NR3C1* levels in control wells. Bars represent mean ± SEM of triplicate wells; * indicates $p < 0.05$ versus control wells; # indicates $p < 0.05$ silibinin+ dexamethasone versus dexamethasone-treated wells.

observed in corticotroph adenomas treated with 17-AAG [12].

Further, silibinin proved capable of reducing ACTH secretion during co-incubation with dexamethasone, regardless of the effect of dexamethasone alone. In fact, among the 5 specimens with clear-cut silibinin-induced decreases in ACTH secretion, sensitivity to negative feedback was variable. Likewise, Riebold et al. [9] had reported that silibinin potentiated the inhibitory effect of dexamethasone on ACTH secretion in 3 specimens and restored sensitivity in 2 dexamethasone-unsuppressed adenomas.

We also investigated the effect of silibinin on *POMC* and *NR3C1* expression in corticotroph adenomas, a finding never previously reported. Silibinin reduced constitutive *POMC* expression in specimens sensitive to the effect of silibinin on ACTH secretion. These results are in line with the effect of silibinin on *POMC*-Luc reporter-transfected AtT-20 cells [9], providing translation of experimental findings to human corticotroph adenomas. *POMC* expression in corticotroph adenomas is known to be variably sensitive to negative steroid feedback [3]; we observed that silibinin reduced *POMC* expression regardless of the effect of dexamethasone alone, either enhancing *POMC* suppression or restoring *POMC* suppressibility. This supports the concept that the HSP90 inhibitor inhibits ACTH synthesis and secretion regardless of inherent sensitivity to negative steroid feedback.

Investigation into the effect of silibinin on glucocorticoid receptor gene expression revealed two-fold consequences: on the one hand, silibinin reduced constitutive glucocorticoid receptor expression, a heretofore unknown effect. This finding suggests that even in the absence of ligand binding, the C-terminal HSP90 inhibitor can modulate gene expression of its client protein, the glucocorticoid receptor. On the other hand, silibinin also reversed dexamethasone-induced inhibition in *NR3C1*; indeed, a marked increase in *NR3C1* expression during dexamethasone co-incubation was observed in

specimens sensitive to silibinin. Transcriptional control of *NR3C1* is known to be complex, with CpG islands, multiple transcription initiation sites, and a large number of promoter transcription factor binding sites including glucocorticoid receptor elements [28, 29]. Of note, the glucocorticoid receptor is subject to autoregulation [28], with both up- and downregulation occurring upon ligand binding [30, 31]. We have previously reported that *NR3C1* expression is reduced during dexamethasone incubation in corticotroph adenomas [4], and the present findings show that silibinin can reverse *NR3C1* inhibition, thereby adding an additional layer of complexity to the action of silibinin. In fact, while no clear-cut increase in glucocorticoid receptor protein was observed in AtT-20 cells treated with silibinin [9], our results suggest that modulation of glucocorticoid receptor gene expression may contribute to the increase in glucocorticoid receptor binding sites, beyond the hypothesized effect on release from the HSP90-GR complex [9].

Of note, both effects on *NR3C1* expression were observed in specimens exhibiting blunted ACTH/*POMC* levels during silibinin incubation. This suggests that, as with other factors associated with steroid-negative feedback, e.g., SMARCA4 (formerly BRG1), HDAC2 [32], 11BHSD2 [33], and NR3C1 itself [4, 34], HSP90 abundance may vary among corticotroph tumors. Indeed, levels of the HSP90 alfa and beta proteins differed among Cushing's disease specimens [9], thereby providing support for different sensitivities to the HSP90 inhibitor.

Silibinin has been shown to act differently from other HSP90 inhibitors, presumably by virtue of its interaction with the C-terminal domain [35] rather than the N-terminal, ATP-binding domain, as occurs for geldamicin derivates such as 17-AAG or pyrazole-containing compounds such as CCT018159 [36]. In fact, comparison of the effects of silibinin and 17-AAG in the mouse corticotroph cell line revealed that the N-terminal HSP90 inhibitor induced glucocorticoid receptor protein degradation, abolished dexamethasone binding, and reduced glucocorticoid receptor transcriptional activity [9]. Conversely, silibinin increased the number of glucocorticoid receptor binding sites and glucocorticoid receptor transcriptional activity, which translated into increased dexamethasone-induced POMC transrepression and blunted ACTH levels [9].

In connection, Shan [12] observed that 17-AAG appeared to reduce *POMC* transcription to a greater extent in *USP8*-variant corticotroph adenomas compared to wildtype adenomas. In the present series, only 2 adenomas carried somatic *USP8* variants; from a descriptive point of view, responsivity to silibinin was observed in both *USP8*-variant and wildtype adenomas. Somatic mutations in the deubiquitinase *USP8* gene occur in some 30% of corticotroph adenomas and have been associated with EGF receptor signaling [37, 38], intracellular ACTH turnover [18], and, most interestingly, increased nuclear HSP90 staining [39]. 17-AAG suppressed EGF receptor protein in a hepatic cell line [12]; the link between HSP90 inhibitors and the EGF receptor pathway in corticotroph adenomas remains to be established.

In conclusion, our study expands upon previous findings with the natural HSP90 inhibitor, silibinin. We showed that silibinin can reduce both spontaneous and steroid-modulated ACTH synthesis and secretion and directly affects glucocorticoid receptor gene expression in human corticotroph adenomas. Some adenomas proved exquisitely sensitive to the inhibitory effect of silibinin, paving the way to individualized, target therapy.

Statement of Ethics

The study protocol (ID # 02C102) was reviewed and approved by the Ethical Committee of the Istituto Auxologico Italiano, on April 12, 2011. Written informed consent for secondary use of surgical tissues was obtained at referring neurosurgical centers.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Methodology and validation, and formal analysis, Maria Francesca Cassarino, Antonella Sesta, and Francesca Pecori Giraldi; data curation, Francesca Pecori Giraldi, Marco Losa, and Giovanni Lasio; writing – draft preparation, review, and editing, Francesca Pecori Giraldi and Marco Losa; supervision, Francesca Pecori Giraldi. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Raw data are deposited at https://doi.org/10.13130/RD_UNIMI/QBRJNN. Further inquiries can be directed to the corresponding author.

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