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SHORT COMMUNICATION

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Effect of the multidrug resistance modulator valspodar on serum cortisol levels in rabbits

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Abstract *Purpose*: To contribute to a better understanding of the physiological role of P-glycoprotein (P-gp) in the adrenal gland, we initiated our studies in rabbits. The aim of our study was to explore the effect of the selective multidrug resistance (MDR) modulator PSC 833 (valspodar) on serum cortisol in rabbits. *Methods*: Baseline and corticotropin-stimulated serum cortisol levels were measured before and after valspodar treatment in adult male rabbits. Seven rabbits were treated with 50 mg/kg per dose and seven, with 75 mg/kg per dose of valspodar subcutaneously. Serum cortisol levels were determined by radioimmunoassay adjusted for expected values. *Results*: Serum cortisol levels (baseline as well as corticotropin-stimulated) increased after both valspodar treatment regimens. The increase was dose-dependent and was higher for the baseline than for the corticotropin-stimulated values. Serum valspodar levels exceeding 1000 ng/ml were achieved in all except one animal in each group. We hypothesize that the increased serum cortisol levels were due to increased adrenocorticotropic hormone (ACTH) secretion after valspodar treatment, but, unfortunately, we could not measure ACTH properly in rabbits by means of the commercially available kits. *Conclusions*: Our study indicates that P-gp is not involved in steroid hormone secretion in the adrenal gland. This is evident from observations that serum cortisol levels were found to have increased rather than decreased in rabbits treated with a P-gp blocker and that the treated animals appeared healthy and normal. Since P-gp was found to play an important role in protection against xenobiotics in some other organs, further studies to explore the protective role of P-gp in the adrenal gland are warranted.

Key words Cortisol · Multidrug resistance · P-glycoprotein · PSC-833 (valspodar)

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Introduction

P-glycoprotein (P-gp) is a 170-kDa transmembrane protein that was originally found to be involved in the efflux of anticancer drugs from tumor cells [12]. This protein is a major contributor to the multidrug resistance (MDR) of cancer cells, although it is also present in a variety of normal tissues in mammals [11, 13, 22].

The function of P-gp can be modified by a number of drugs (MDR modulators) that bind to P-gp [19]. Information about the increased effectiveness of cytotoxic treatment gained by using these modulators, at least in solid tumors, is lacking. However, several studies concerning the pharmacokinetics of various drugs have clearly shown that the drugs' properties are altered significantly by MDR modulators [2, 4, 7–10, 15, 25]. They increase the AUC (area under the curve) and, correspondingly, the toxic side effects of the MDR-related drugs by blocking P-gp-mediated excretion of the drug through the kidney and liver as well as by other mechanisms such as inhibition of the other transporters and cytochrome P-450.

Thus far, the knowledge of the physiological function of P-gp has been derived from studies on P-gp "knock-out" mice [5, 16, 17]. In contrast to humans, mice have two *mdr* genes (*mdr1* and *mdr2*), which together may fulfil the same function as the *mdr1* gene in humans. Studies on mdr1a (−/−) knock-out mice, in which a complete lack of P-gp in the intestinal epithelium and in brain endothelial cells was found, confirmed the important role of P-gp in the excretion of xenotoxins from the body [17], in the absorption of hydrophobic drugs

from the gastrointestinal tract [5], and in sustaining the blood-brain barrier [16]. On the basis of these observations, the most important role of P-gp seems to be the protection of organisms from toxic xenobiotics.

The physiological role of P-gp in the adrenal gland cortex, in which it has been found to be expressed at high levels in humans as well as other mammals [20, 21], has not yet been explored. Steroid hormones interact with P-gp at high concentrations and are capable of reversing drug resistance in P-gp-expressing tumor cells [14, 23, 24, 26]. In vitro studies have also shown that some steroid hormones, such as cortisol, dexamethasone, and aldosterone, are transported by P-gp [14, 23, 24], whereas others, such as progesterone, do not appear to be transported by P-gp [14, 23], although they can bind to P-gp and antagonize the binding and P-gp-mediated transport of other substances. Both properties seem to vary according to the steroid hydrophobicity and the phosphorylated state of P-gp [1]. To our knowledge, there is no in vivo study available. According to the observation made by Borst and coworkers [5], the double (*mdr1a* and *mdr1b*)-knock-out mice in which a complete lack of P-gp in the adrenal glands was found were apparently healthy and fertile under laboratory conditions. Nevertheless, it should be pointed out that this observation is very limited, and we have no information about the condition of those mice in a stressful situation.

To contribute to a better understanding of the physiological role of P-gp in the adrenal gland, we initiated our studies in rabbits. We established that P-gp is highly expressed in the adrenal cortex of the rabbits [6]. Surprisingly, in our previous study [6], treatment with the MDR modulator cyclosporine A (CsA) resulted in elevated rather than diminished serum cortisol levels in rabbits. The present study was initiated to explore further this interesting and, to some extent, surprising observation. The aim of our study was to explore the effect of a more potent and more selective MDR modulator [3], PSC 833 (valspodar), on serum cortisol as well as adrenocorticotropic hormone (ACTH) levels in rabbits and, using these data, to examine the adrenal gland function as well as the hypothalamic-pituitary-adrenal axis during treatment.

Material and methods

Animals and treatment

New Zealand White adult male rabbits were housed in laboratory conditions on a standard pellet diet and tap water. In 14 rabbits, baseline serum cortisol and ACTH levels were measured at 8 a.m. on day 1, then stimulation by corticotrophin was performed and cortisol levels were measured at 1 and 8 h after stimulation. β^{1-24}-Corticotrophin (0.5 mg i.m.; Synachten Depot, Ciba-Geigy, Basel, Switzerland) was used for adrenal stimulation. After an interval of 12 days the rabbits were treated with valspodar (Novartis Pharma AG, Basel, Switzerland). Valspodar 1-ml ampules contain 50 mg PSC 833 dissolved in 60% Cremophor-EL. Two doses of valspodar were injected subcutaneously at 6 p.m. on day 13 and at 6 a.m. on day 14. Seven rabbits were treated with 50 mg/kg per dose and seven, with 75 mg/kg per dose of valspodar. Baseline serum cortisol and ACTH levels were measured at 8 a.m. and corticotrophin-stimulated cortisol levels were measured at 1 h and 8 h after stimulation on day 14. The serum valspodar levels were measured at 8 a.m., 9 a.m., and 4 p.m. on day 14. Blood samples were collected by cardiac puncture.

In seven rabbits initially treated with the high dose of valspodar treatment with the vehicle was initiated after an interval of 12 days. Vehicle control treatment was performed at the same intervals and with the same volume of injected substance used for the valspodar treatments. The saline control treatment was not repeated since in our previous study [6] using CsA as an MDR modulator there was no difference in either basal or stimulated cortisol levels after saline treatment.

Cortisol and ACTH assays

Serum cortisol levels were determined by radioimmunoassay (CORT-CT; CIS Biointernational, Gif-sur-Yvette, France) adjusted for expected values. The PSC 833 RIA-kit (Novartis Pharma AG) was used for serum valspodar level determination and the radioimmunoassay was optimized for analyses of serum. For ACTH determination, two immunoradiometric assays (ACTHK-PR and ELSA-ACTH; CIS Biointernational) were used. The ACTH-PR kit recognizes amino acid sequences 1–24 of the N-terminal part of ACTH, which is common to various species, and the antiserum is raised in rabbits. ELSA-ACTH is a two-sided immunoradiometric assay that recognizes the N-terminal and C-terminal of 39 amino acids containing human ACTH. The accuracy of ACTH determination was checked with the use of a hypoglycemia test. Blood glucose, serum cortisol, and ACTH values were measured prior to and at 45 and 90 min after the onset of insulin (porcine crystalline insulin at 3 units/kg)-induced hypoglycemia.

Results

Serum cortisol levels (baseline as well as corticotropin-stimulated) increased after both 50-mg/kg and 75-mg/kg valspodar treatments (Tables 1, 2). The increase was dose-dependent and was higher in animals treated with the high dose of valspodar. However, the baseline serum cortisol increase was higher than the corticotropin-stimulated increase in both groups of animals, regardless of the dose of valspodar used.

Serum cortisol levels were not affected after vehicle treatment. In seven animals the mean serum cortisol baseline level was 12.14 (SD 5.40) nmol/l before and 16.57 (SD 13.6) nmol/l after vehicle treatment. The mean value recorded for serum cortisol at 1 h after corticotropin stimulation was 37.57 (SD 8.28) nmol/l before and 49.86 (SD 15.18) nmol/l after vehicle treatment, whereas the values noted at 8 h after corticotropin stimulation were 72.86 (SD 18.07) and 88.43 (SD 16.46) nmol/l, respectively.

Serum valspodar levels tended to increase with increasing drug dose, although the levels varied greatly from one experimental animal to another (Tables 3, 4). The mean serum valspodar levels determined in seven animals treated with the low dose were 2051 and 1620 ng/ml at 2 and 10 h after the last injection of the drug, respectively, whereas the levels determined in the seven animals treated with the high dose were 1976 and

Table 1 Serum cortisol levels determined before and after valspodar treatment in rabbits (*c.s.* corticotropin stimulation)

Animal number	Before treatment (nmol/l)			After 2 × 50 mg/kg valspodar (nmol/l)		
	Baseline	1 h after c.s.	8 h after c.s.	Baseline	1 h after c.s.	8 h after c.s.
1	10	19	31	22	26	31
2	12	32	58	27	61	80
3	12	16	50	7	30	65
4	10	13	12	6	20	28
5	9	45	108	16	39	32
6	11	18	50	31	51	85
7	9	26	50	32	37	38
Mean value	10.4	24.1	51.3	20.1	37.7	51.3
SD	1.27	11.21	29.49	10.79	14.32	24.66
t-test[a]				$P = 0.0583$	$P = 0.0374$	$P = 1.0$

[a] Matched-pair *t*-test

Table 2 Serum cortisol levels measured before and after valspodar treatment in rabbits (*c.s.* corticotropin stimulation, *ND* not done)

Animal number	Before treatment (nmol/l)			After 2 × 75 mg/kg valspodar (nmol/l)		
	Baseline	1 h after c.s.	8 h after c.s.	Baseline	1 h after c.s.	8 h after c.s.
1	10	31	76	31	137	ND
2	7	34	82	60	122	130
3	11	31	35	20	68	ND
4	8	41	85	58	76	124
5	17	42	87	55	47	116
6	10	31	79	22	53	82
7	22	53	66	98	102	110
Mean value	12.14	37.57	72.86	49.14	86.43	112.40
SD	5.40	8.28	18.07	27.49	34.61	18.62
t-test[a]				$P = 0.0072$	$P = 0.0115$	$P = 0.0155$

[a] Matched-pair *t*-test

Table 3 Serum valspodar levels measured after 2 × 50-mg/kg treatment in rabbits

Animal	2 × 50 mg/kg valspodar (ng/ml)		
	8 a.m.	9 a.m.	4 p.m.
1	2754	1693	1732
2	1301	1232	1436
3	2992	2288	225
4	1897	1395	3539
5	3638	3320	1668
6	1203	952	1797
7	573	310	945
Mean value	2051.1	1598.6	1620.3
SD	1109.7	974.8	1013.6

Table 4 Serum valspodar levels measured after 2 × 75-mg/kg treatment in rabbits (*ND* not done)

Animal	2 × 75 mg/kg valspodar (ng/ml)		
	8 a.m.	9 a.m.	4 p.m.
1	2756	3102	ND
2	2793	2068	5900
3	2381	2052	ND
4	1801	1207	1155
5	2187	1422	2523
6	994	798	1419
7	920	701	454
Mean value	1976.0	1621.4	2290.2
SD	774.0	847.9	2150.8

2290 ng/ml, respectively. Levels exceeding 1000 ng/ml were achieved in all except one animal in each group.

ACTH levels determined by ACTH-PR immunoassay using antiserum raised in rabbits were extremely high. On the other hand, ACTH levels determined by ELSA-ACTH immunoassay were extremely low. Therefore, we checked both tests with the hypoglycemia test. After insulin administration the mean blood glucose level in rabbits decreased from 8.6 to 2.4 mmol/l and the mean cortisol level increased from 10.6 to 61.0 nmol/l, whereas ACTH levels remained constantly high when measured by ACTH-PR immunoassay and stayed constantly low when measured by ELSA-ACTH immunoassay.

Discussion

Treatment with the P-gp modulator valspodar resulted in increased serum cortisol levels in rabbits. The increase was higher for baseline values than for corticotropin-stimulated values and was found to be dose-dependent.

These findings are in concordance with the results obtained in our previous study, which was performed with CsA, a less potent and less selective P-gp modulator [6].

Serum valspodar levels were highly variable among rabbits and there was a tendency toward higher levels in the group of animals treated with the higher dose. Levels exceeding 1000 ng/ml, which are required for optimal MDR modulation in vitro [1], were achieved in six of seven rabbits in both treatment groups. However, in the remaining two rabbits, whose serum PSC-833 levels were not high enough for MDR modulation, serum cortisol levels increased as well, which may indicate that the increased serum cortisol levels were not related to MDR modulation.

Unfortunately, we could not measure ACTH properly in rabbits by means of the commercially available kits. The values were extremely and uniformly high when we used the radioimmunoassay containing an antiserum raised in rabbits, and ACTH levels were extremely and uniformly low when radioimmunoassay with the monoclonal antibodies to the N- and C-terminal parts of human ACTH was used. As we were incapable of measuring ACTH levels, we could not evaluate a possible effect of PSC 833 treatment on the pituitary ACTH cells. Our experience clearly points out the difficulties associated with studies on the physiological function of P-gp; in humans these studies are limited by the toxic side effects of MDR modulators, whereas in animal studies, many methodological problems must be overcome before we can expect to achieve some conclusive results.

We hypothesize that the increased serum cortisol levels observed after valspodar injection as well as after CsA treatment [6] might have been due to increased ACTH secretion. Our beliefs are based on the observation that the increase in baseline cortisol levels was higher than the increase in corticotropin-stimulated cortisol values. We believe that the pre-existing endogenous ACTH levels were so high that additional exogenous corticotropin could not further stimulate cortisol synthesis in the adrenal gland. Furthermore, the increase in serum cortisol levels was higher at 1 h after corticotropin stimulation, when presynthesized cortisol is released, than at 8 h after corticotropin stimulation, when de novo-synthesized cortisol is released. This shows that the endogenous ACTH levels, which had been stimulating the synthesis of the stored cortisol, were higher after valspodar treatment. Such an increase in baseline cortisol levels, as observed in our experimental animals, could not be achieved by a drug-mediated increase in cortisol synthesis or an inhibition of cortisol catabolism because of the feedback mechanisms regulating the pituitary-adrenal axis. In vitro data also support our speculation. Sheppard [18] found CsA as well as other immunophilin ligands to be potent stimulators of proopiomelanocortin-derived peptide (e.g., ACTH) secretion from corticotrope cells. Immunophilin ligands act through inhibition of calcineurin, leading to increased calcium influx and, consequently, enhanced ACTH exocytosis. Although PSC-833 does not bind to cyclophilins, this does not rule out its possible interaction with calcineurin, which should be explored further.

Although in vitro studies have shown that steroid hormones are substrates for P-gp and, furthermore, that they can even be transported by P-gp [1, 14, 23, 24, 26], our study indicates that P-gp is not involved in steroid hormone secretion in the adrenal gland. This is evident from the observation that the serum cortisol levels were found to have increased rather than decreased in rabbits treated with a P-gp blocker. Moreover, the treated animals appeared healthy and normal. This observation is in concordance with the results obtained in double-knock-out mice by Borst et al. [5].

Although the physiological function of P-gp has not yet been fully explored, it has been found to have a pivotal role in protecting organisms from toxic xenobiotic compounds. This is achieved by the excretion of these compounds through the genitourinary, biliary, and intestinal tracts and by prevention of their accumulation in critical organs such as the brain and testis. On the basis of these observations, it could be presumed that the role of P-gp in the adrenal gland is a protective one. Therefore, further studies to explore the protective role of P-gp in the adrenal gland are warranted.

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