Reproductive endocrine side effects of antiepileptic drugs

Student Thesis

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Contents

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Abstract

RATIONALE: There is a complex interplay between epilepsy, antiepileptic drugs, and reproductive endocrine disorders. Epilepsy itself may influence on the hormonal balance, hormones may affect epilepsy, hormones may influence on the efficacy and metabolism of antiepileptic drugs and antiepileptic drugs may alter the hormone levels.To better understand the interaction of antiepileptic drugs and production of steroid hormones, we investigated possible effects of valproate, carbamazepine and levetiracetam using the H295R steroidogenesis model.

METHODS: H295R human adrenal carcinoma cells are capable of full steroidogenesis. Cells were exposed to three different antiepileptic drugs (valproate, carbamazepine and levetiracetam) for 48 hr. Medium was collected and analyzed for hormone production (estradiol, testosterone, progesterone and cortisol).

RESULTS: We observed a significant, dose-dependent reduction in the production of estradiol in cells exposed to valproate (300 – 1500 μM). At a drug concentration of 600 μM, estradiol levels were reduced to 40% compared with controls. Testosterone levels were not significantly changed. This resulted in a dose-dependent increase of the testosterone to estradiol ratio. No significant dose-response effect on hormone production was seen with carbamazepine or levetiracetam.

CONCLUSIONS: H295R cells exposed to valproate, carbamazepine, or levetiracetam showed different patterns of hormone production. The most prominent finding was a significant dose-dependent increase of the testosterone/estradiol ratio in cells exposed to valproate. This suggests valproate is capable of inhibiting conversion of testosterone to estradiol. No effects on sex steroid hormone production were observed with carbamazepine or levetiracetam.

Introduction

There is a higher occurrence of reproductive endocrine disorders among both men and women with epilepsy than in the general population (Herzog et al. 1986a, 1986b, Bilo et al.1988, Isojärvi et al. 1993, Löfgren et al. 2006) and fertility is known to be reduced (Webber et al. 1980, Schupf et al. 1994, 1996, Artama et al. 2006). In a large population-based study from 1998, Wallace et al. revealed that fertility rates among women in the general population were 33% higher than among women with epilepsy. In a retrospective questionnaire study of a cohort of female outpatients aged 18-45 performed by Svalheim et al. (2003), menstrual disorders were found to be more frequent in a group of 265 women with epilepsy (48.0 %) than in the matched controls without epilepsy (30.7%). Herzog et al. (1986a) have reported that diminished libido and potency are unusually common among men with epilepsy.

There is a complex interplay between epilepsy and reproductive hormones. Epilepsy itself may influence on the hormonal balance, hormones may affect epilepsy, hormones may influence on the efficacy and metabolism of antiepileptic drugs (AEDs) and AEDs may alter the hormone levels. The increase in endocrine disturbances among patients with epilepsy has been attributed both to epilepsy itself and to the use of AEDs (Isojärvi et al. 2005).

In this thesis I will first give a general overview of some of the research concerning the reproductive endocrine effects of epilepsy, the effects of reproductive hormones on epilepsy, the effects of epilepsy on AEDs and reproductive endocrine effects of AEDs. Finally I will present an *in vitro* study of the effects of valproate (VPA), carbamazepine (CBZ) and levetiracetam (LEV) on steroidogenesis, using a human adrenal adenocarcinoma cell-line, the H295R cells.

Reproductive endocrine effects of epilepsy

The association between epilepsy and reproductive disorders is not fully known. In general, reproductive endocrine disorders and infertility often result from an alteration of the hypothalamo-pituitary gonadal axis. Epilepsy itself may induce a dysfunction in this system and thereby affect the gonadal function (Herzog et al. 2003, Fawley et al. 2006). Disturbances of the hypothalamo-pituitary gonadal axis may be initiated by higher cortical systems, and the temporo-limbic structures seem to be especially important (Herzog 1989). In 2003 Herzog

and co-workers evaluated reproductive endocrine function in women with unilateral temporo-limbic epilepsy (TLE) and compared it with the reproductive endocrine function in normal control subjects. Menstrual disorders were found to be more common among women with TLE than among the healthy controls and reproductive endocrine disorders were overrepresented among the women with TLE (27.8%) in comparison to the expected frequency in the general population (8.0%). One of these disorders is polycystic ovary syndrome (PCOS) which is a condition containing at least two out of three criteria: 1: oligo- and/or anovulation 2: clinical and/or biochemical signs of hyperandrogenism 3: polycystic ovaries. In this article it is stressed that the suggestion that epileptiform discharges may disrupt the temporo-limbic modulation of hypothalamo-pituitary function is supported by the close relationship between the paroxysmal discharges and by the occurrence of the abnormal changes in LH-pulse frequency and prolactin levels. Moreover, the findings of abnormal elevations of prolactin following right lateral discharges and the greater frequency of LH pulse suppression followed by elevation of mean baseline LH secretion after left lateral discharges, also support the validity of this mechanism. These findings also suggest that there is a lateralized asymmetry of neuroendocrine control (Herzog et al. 2003). Moreover, an animal study on female rats has found that ipsilateral activation of the amygdala activated neurons in areas of the hypothalamus that are known to be involved in reproductive endocrine function (Silveira et al. 2000).

In several studies, Herzog et al. have found reduced levels of bioactive testosterone in men with localization-related epilepsy in comparison to normal controls and premature aging of the reproductive system did seem to occur more often in the men with epilepsy (Herzog el al. 2004, 2005). Moreover, several clinical studies report that certain reproductive disorders seem to be associated with the laterality of the seizure. Several findings indicate that endocrine dysfunction and PCOS is associated with left-sided temporo-limbic epilepsy and that hypogonadotropic hypogonadism is more commonly found in women with right-sided focus (Herzog et al. 1986a/b, 1990, 1993, 2003). It has also been suggested that for men with epilepsy, reproductive and sexual dysfunction is associated with right-sided temporal lobe paroxysmal discharges (Herzog et al. 1990).

Morrell and co-workers studied the sexual response in nine men and eight women with temporal lobe epilepsy compared with normal controls. They measured the genital blood flow (GBF) and digital pulse when the participants watched sexual neutral and erotic videos and subjective ratings were obtained. Men and women with epilepsy reported fewer sexual experiences than the controls. The mean increase in GBF was 184% in men with temporal

lobe epilepsy versus 660% in controls and 117% in women with temporal lobe epilepsy versus 161% in controls (Morrell et al. 1994).

Several animal experiments have found that amygdala kindling may affect the hormonal balance. One study in female rats has shown that seizures initiated in the amygdala lead to an impairment of the hypothalamo-pituitary axis, resulting in anovulation (Edwards et al. 1999a). Moreover, an animal experiment in male rats has revealed that both focal limbic seizures and generalized seizures disturbed normal reproductive physiology (Edwards et al. 1999b). Feeney and co-workers have studied the occurrence of hyposexuality in male cats with temporal lobe epilepsy. They produced focal epilepsy in the animals by unilateral injection of aluminum hydroxide either into the basolateral amygdala or the anterior sigmoid gyrus. Hyposexuality was detected in the temporal lobe group. Neither the motor cortex group nor the normal control group showed any signs of sexual dysfunction (Feeney et al. 1998).

Reproductive hormones can affect epilepsy

It is well known that endocrine disorders and hormonal fluctuation may have an effect on seizure frequency and on epilepsy itself. Catamenial epilepsy is common in women with epilepsy. In these women, the occurrence of seizures fluctuates with the menstrual cycles.

In a clinical study including 184 women with intractable complex partial seizures Herzog and co-workers suggested the occurrence of three different patterns of catamenial epilepsy; first a pattern with perimenstrual seizure exacerbation, secondly a pattern with preovulatory seizure exacerbation and third a pattern with seizure exacerbation throughout the second half of inadequate luteal phase cycles in women with non-ovulatory cycles. In this material 71.4% of the women with ovulatory cycles and 77.9% of the women with inadequate luteal phase cycles were found to have seizure exacerbations in relation to one of these three patterns (Herzog et al. 1997). Similar results have been detected by Taubøll and co-workers who found a positive cross-correlation between seizures and menstrual cycles in seven of nine women in a study based on seizure dairies (Taubøll et al. 1991).

The research in this area has mainly focused on the possible effects of estrogen and progesterone and it has been suggested that estrogen has excitatory and progesterone has inhibitory effects on neuronal excitability and seizure frequency (Holmes and Donaldson 1987). It has been shown that progesterone and several of its metabolites bind to a site on or near the GABA-receptor complex and act agonistic (Majewska et al. 1986). Moreover,

Taubøll and co-workers have studied the anticonvulsant effect of progesterone and its brain metabolite 3α-OH-DHP both *in vitro* and *in vivo*. An *in vitro* study of the anticonvulsant effect of 3α-OH-DHP compared with phenobarbital in rat hippocampal slices revealed that the progesterone metabolite was nearly twenty times as potent as phenobarbital in enhancing recurrent inhibition (Taubøll and Gjerstad 1993). In an *in vivo* study concerning the effect of 3α-OH-DHP and progesterone on focal epileptic seizures in the cat's visual cortex both had a dose-dependent effect on the increase in seizure threshold, 3α-OH-DHP being about 20 times as potent as the latter (Taubøll and Lindström 1993).

One of the first and most extensive studies to report the cyclic fluctuation of seizure frequency was published by Laidlaw in 1956. He followed 50 women with generalized tonic-clonic seizures through 934 patient-years and revealed that 72% had an exacerbation of seizures in relation to the menstrual cycle. In this paper, Laidlaw suggested that progesterone might exert an anticonvulsant action, and that the withdrawal of progesterone might lead to seizure exacerbations (Laidlaw 1956).

In 1959 Logothetis and co-workers suggested that exacerbation of epileptic seizures just before the time of menstruation could be explained by a convulsive effect of estrogen and not by the withdrawal of progesterone. To support this theory they gave intravenous injections of the estrogenic substance premarin to 16 epileptic patients and found that 11 showed an increased epileptogenic activity. Moreover, they studied the possibility of using progesterone in treatment of epilepsy by giving five of the patients with catamenial epilepsy 10 mg progesterone three times a day for four to six days before menstruation. With a follow-up of six months, three of the patients had improved. In the same article they also present an animal experiment consisting five rabbits with epileptic lesions and one with an intact cortex. They detected a significant epileptogenic effect of premarin both when applied over an irritable cortical lesion and injected intravenously (Logothetis et al. 1959).

In 1976 Bäckström investigated nine periods in seven women with partial epilepsy in respect to frequency of seizures and levels of estrogen and progesterone. Six of the cycles were ovulatory, and in these he detected a positive correlation between the number of secondary generalized seizures and the mean estrogen/progesterone ratio and a negative correlation to plasma progesterone levels. Three of the cycles were non-ovulatory and in these he found an increase in the number of seizures during days of high estrogen. Moreover, the same research group has studied this further by giving seven women with partial epilepsy progesterone intravenously until the levels reached plasma concentration as during the luteal

phase. Four of the women showed a significant decrease in spike frequency during the infusion (Bäckström et al. 1984).

Reproductive hormones can influence on AEDs

It has been showed that oral contraceptive pills (OCP) may reduce the plasma levels of lamotrigine (LTG). In 2001 Sabers et al. illuminated this through seven case studies of women using both LTG and OCP. They found a mean reduction in LTG plasma levels of 49 % when OCP were taken in addition. These findings were supported in a retrospective study from 2003 where they measured LTG plasma levels in 22 women also taking OCP and compared these values with the LTG plasma levels in 30 women not taking OCP. They found a more than 50% reduction in LTG plasma levels in the women who took OCP in addition compared with those who only took LTG (Sabers et al. 2003).

These findings were supported by Sidhu et al. (2005) in a prospective study containing 16 healthy subjects taking both OCP and LTG in 105 days and then LTG alone in the following three weeks. They detected an average decrease in maximum observed serum concentration of LTG of 39% and a 52% decrease in area under the serum concentration-time curve during co-administration with OCP.

Moreover, a case report of a 26-year old girl with epilepsy indicates that OCP might have an effect of the serum levels of VPA. In this report a clear reduction in VPA-levels were measured when she was taking the active OCP and it was seen an increase in seizure frequency in the same time period (Herzog et al. 2005).

It has also been suggested that the serum concentrations of LEV can be affected of reproductive hormones. A recent retrospective study on the serum concentration/dose ratio of LEV in 20 women before, during and after pregnancy detected a significant decline of LEV concentrations during the third trimester and an increase rapidly after delivery (Westin et al. 2008).

Reproductive endocrine effects of AEDs

Research in recent years has shown that a number of different AEDs can affect reproduction (Isojärvi et al. 2005). In a population based cohort study on men and women

with epilepsy in Finland, Artama and co-workers found the reproductive rates to be reduced in both male and female patients on AEDs, most prominent in men (Artama et al. 2006).

Several common AEDs (e.g. phenobarbital, phenytoin and carbamazepine) induce the hepatic CYP 450 dependent steroid hormone breakdown and production of sex-hormone binding globulin (SHBG). This may result in decreased levels of biologically active sex hormone serum concentrations (Isojärvi et al. 1995, Rättyä et al. 2001, Herzog 2004, 2005, 2006). Diminished levels of bioactive testosterone and estradiol may result in diminished potency in some men and menstrual disorders in some women and may also lead to reduced fertility. It is also well known that liver enzyme-inducing AEDs (EIAEDs) might reduce the efficacy of OCP by enhancing their metabolism.

It is well documented that VPA has an effect on the reproductive hormonal balance in both men and women. VPA does not induce liver enzymes, and the mechanism by which VPA alters reproductive endocrine function is not fully known. VPA has been shown to be an inhibitor of the enzyme microsomal epoxide hydrolase (mEH) (Kerr et al. 1989, Bernus et al. 1997). Moreover, it has been demonstrated that the inhibition of this enzyme suppresses the conversion of testosterone to estradiol (Hattori et al. 2000). In addition VPA is known to be a histone deacetylase (HDAC) inhibitor. These molecules inhibit HDAC activities which may lead to histone hyperacetylation, change of chromatin structure, and removal of transcriptional corepressors and thus induce transcription of many genes (Chen et al. 1997, 2007). Chen and co-workers have showed that valproic acid suppressed the expression of CYP11A1 and decreased steroid secretion by increasing the ubiquitination and degradation of steroidogenetic factor 1 (SF-1), an important factor in transcription of all steroidogenic genes (Chen et al. 2007). Moreover, it has been suggested that VPA inhibits aromatase activity and thereby leads to an increased testosterone to estradiol balance (Gregoraszczuk et al. 2000, Taubøll et al. 2002, 2003, 2006, Jacobsen et al. 2008).

In the present study we exposed the H295R human adrenal adenocarcinoma cells to VPA, CBZ and LEV, and in the following part a more detailed presentation of reproductive endocrine effects of these drugs is given.

Reproductive endocrine effects of valproate

Women

A large amount of studies have revealed that use of VPA may cause hyperandrogenism, menstrual disturbances and polycystic ovaries in women (Isojärvi et al. 1993, 1996, 2001, 2005, Löfgren et al. 2006, Morrell et al. 2003, Svalheim et al. 2003). In 1993 Isojärvi and co-workers performed a cross-sectional study on a population of 238 women with epilepsy concerning reproductive endocrine disturbances. They revealed that menstrual disturbances occurred in 45 % of the women taking VPA for epilepsy, and these disorders were frequently associated with polycystic ovaries and/or hyperandrogenism. Polycystic ovaries and hyperandrogenism were especially common in women treated with VPA before the age of 20, and in this group one or both of these conditions were found in 80% of the patients. Moreover, serum androgen levels (testosterone, free testosterone and DHEAS) were found to be increased in women taking VPA. In a follow-up study from 1996, these endocrine symptoms were associated with obesity, and weight gain, hyperinsulinemia and low serum insulin-like growth factor binding protein 1 (IGFBP-1) concentrations were suggested to be important pathogenetic factors. Polycystic ovaries, hyperandrogenism or both were found in 64 % of the women treated with VPA, and these women had higher serum testosterone and insulin levels and lower serum SHBG and IGFBP-1 levels and they tended to be more obese than the VPA treated women without polycystic ovaries and hyperandrogenism. However, polycystic ovaries and hyperandrogenism were also seen in the lean women receiving VPA (Isojärvi et al. 1996). A study of women from Finland, Norway and the Netherlands supports these findings. The frequency of polycystic ovaries and/or hyperandrogenism was found to be 79% in obese women on VPA and 65 % in lean women on VPA. The obese women with polycystic ovaries, hyperandrogenism or both had hyperinsulinemia and an unfavorable serum lipid profile (Isojärvi et al. 2001).

In a cross-sectional study including 233 patients, Morrell and co-workers found that compared with LTG in monotherapy, VPA in monotherapy was associated with weight gain and higher androgen levels in women with epilepsy. The elements they looked at in this study were endocrine and lipid measures during the early follicular phase of the menstrual cycle, prevalence of menstrual disorders and bodyweight (Morrell et al. 2003). These findings were supported by Stephen et al. (2001) in a clinical study where they found no alterations in

reproductive hormones in women on LTG in monotherapy, whereas the women on VPA in monotherapy had significantly higher testosterone levels. Moreover, it has been observed in patients that VPA induced obesity, hyperinsulinemia, lipid abnormalities and polycystic ovaries/hyperandrogenism can be reduced by substituting LTG for VPA (Isojärvi et al. 1998).

Several *in vitro* and animal studies have supported the clinical findings concerning the possibility of an unfavorable effect of VPA on reproductive endocrine function. *In vitro* studies on porcine ovarian follicular cells have showed that VPA added to the cell culture medium caused a significant effect on steroidogenesis in both non-stimulated and gonadotropin-stimulated cells and an inhibition of the conversion of testosterone to estradiol seen as an increase in the testosterone to estradiol ratio (Gregoraszczuk et al. 2000, Taubøll et al. 2003). Moreover, Taubøll et al. (2006) have studied the effect of VPA and LEV on prepubertal porcine ovarian follicular cells and found that VPA altered both basal gonadotropin-stimulated testosterone and estradiol secretion with an increase in testosterone levels and a decrease in estradiol levels while LEV only affected steroid secretion under basal conditions when gonadotropins were not added. The effect of VPA on the follicular steroidogenesis has been found to be irreversible *in vitro* (Taubøll et al. 2002). Moreover, a number of studies on gonadal function and reproductive hormones in non-epileptic rats have demonstrated that long-term VPA treatment induces changes in ovarian morphology by increasing the number of ovarian cysts and altering sex-steroid hormone levels (Taubøll et al. 1999, Røste et al. 2001, 2001). These findings would indicate that the endocrine alterations observed in women taking VPA for epilepsy is caused by the medication rather than epilepsy itself. This suggestion is strongly supported similar findings in non-epileptic women taking VPA for bipolar mood disorders (O`Donovan et al. 2002).

Men

It has also been found that VPA may cause endocrine disturbances in men. Rättyä et al. (2001) have showed that VPA increases serum androgen concentration in men with epilepsy. Røste and co-workers have performed an animal study regarding possible morphological changes in the testes of male Wistar rats treated with VPA. 15 rats were fed with a low dose of VPA mixture, 20 rats were fed with a high dose of VPA mixture and 15 rats were fed with a control solution twice a day for 90 days. They detected a highly significant decrease (51%) in testicular weight in the high-dose group and no changes in the other groups (Røste et al. 2001). Moreover, the same research group has investigated possible

drug-specific effects on semen quality and testicular size in men on long-term treatment with either VPA or CBZ and they compared two groups with healthy controls. They did not detect any significant differences between the two treatment groups regarding semen parameters, testicular size, fertility or reported libido. However the VPA-treated patients differed significantly from the controls in having more sperm tail abnormalities and lower testicular size/BMI ratio than the CBZ-treated men (Røste et al. 2003). Isojärvi et al. (2004) have also reported a higher frequency of sperm abnormalities in VPA-treated men compared with normal controls. In addition, the VPA-treated men with abnormal sperm had smaller testicular volumes than the controls. Moreover, Røste et al. (2001) found testicular atrophy and reduced spermatogenesis in male rats treated with VPA. These findings are supported by a recent animal study on adolescent goat bucks fed orally with VPA and compared with a control group. In this study, they found that the treated animals had a higher body weight, a lower testis diameter, a lower plasma level of LH and testosterone leading to a later onset of puberty than the control animals. In addition, the VPA-treated animals had an increased percentage of sperm with DNA damage (Krogenæs et al. 2008).

Reproductive endocrine effects of carbamazepine

Women

Isojärvi and co-workers have performed several prospective and cross-sectional studies concerning the reproductive endocrine effects of CBZ in women with epilepsy (Isojärvi et al. 1990, 1993, 1995, 2001). These studies have all revealed an increase in serum SHBG levels in women treated with CBZ. This increase results in a decreased amount of bioactive estradiol (seen as a diminished estradiol/SHBG ratio) which may be associated with menstrual disorders in some women.

In a cross-sectional study group of 56 women that had received CBZ in monotherapy for more than five years, Isojärvi et al. (1995) found menstrual disorders in 25 %, and they revealed that these disorders in most cases were associated with increased serum estradiol levels and low estradiol/SHBG ratio. However, several studies concerning women taking CBZ for epilepsy do not find an increase in the frequency of menstrual disorders or a larger amount of other reproductive endocrine disorders in these women (Isojärvi et al. 1990, 2001, Murialdo et al 1998).

Moreover, the increase in serum SHBG levels may lead to a reduction of bioactive testosterone, seen as diminished free androgen index (FAI = 100 x serum testosterone/SHBG)

(Isojärvi et al. 1990). This may theoretically be advantageous in women with hyperandrogenism. It has also been revealed that CBZ treatment may lead to a reduction in serum levels of the weak androgen DHEAS in women (Murialdo et al. 1998). The effect of this reduction is not fully known.

Men

CBZ is known to induce the hepatic CYP 450 enzyme system, and this may result in an increase in metabolism of hormones and synthesis of SHBG. This leads to an increased level of serum SHBG and consequently a decreased amount of bioactive testosterone, which is associated with diminished sexual function in some men.

In a prospective long-term study published in 1995, Isojärvi et al. reported no significant change in serum testosterone levels in men treated with CBZ, but they revealed an increase in serum SHBG levels and a decrease in FAI during the five first years of CBZ treatment.

In several clinical studies, Herzog et al. have reported significantly lower levels of serum bioactive testosterone in patients using liver EIAEDs (e.g. CBZ) than in patients on LTG and in controls. Low serum bioactive testosterone is found to be associated with low S-score (sexual interest and function) in these studies. Moreover they revealed a significantly higher level of serum SHBG the patients on EIAEDs than in the other groups (Herzog et al. 2004, 2005, 2006).

Rättyä and co-workers have found low levels of the weak androgen DHEAS and a high concentration of SHBG in men taking CBZ for epilepsy. In the same study, decreased libido, impaired potency or both were reported by 18 % of the male patients on CBZ (Rättyä et al. 2001).

A recent prospective randomised double-blinded withdrawal study revealed that some of the negative effects on reproductive endocrine functions associated with CBZ-treatment may be reversible, even after years of treatment. Men in the withdrawal group demonstrated a significant increase in total testosterone serum levels after withdrawal of CBZ and a non-significant decrease in SHBG concentrations. These changes resulted in a significant increase in FAI in this group (Lossius et al. 2007).

CBZ use may also be associated with reduced sperm motility and increased frequency of morphologically abnormal sperm (Røste et al. 2003, Isojärvi et al. 2004).

Reproductive endocrine effects of levetiracetam

LEV is a new AED with a broad specter of antiepileptic efficacy that may represent an alternative to VPA for many patients. No reproductive endocrine problems have been described in humans using LEV (Taubøll et al. 2006). This is particularly interesting in the treatment of young women, since VPA has been shown to be associated with the development of polycystic ovaries, hyperandrogenism, and menstrual disorders in women (Isojärvi et al. 1993, 2001, 2005, Morrell et al. 2003).

Svalheim et al. (2007) have performed a long-term study on 50 Wistar rats fed with either LEV solution 50 mg/kg, 150 mg/kg or a control solution twice a day for 90-95 days. The treated animals had significantly larger ovaries, a lower number of cysts, and a higher number of corpora lutea and secondary follicles than the untreated animals. This indicates that LEV has a probable effect on the ovaries. Blood tests revealed that the animals treated with LEV had significantly higher testosterone levels and lower levels of estradiol and FSH. Serum progesterone concentrations were higher after LEV-treatment, but the difference was only statistically significant in the low dose group. No significant difference was found in the level of serum LH concentrations. These findings suggest that long term LEV-treatment affects reproductive endocrine function, as well as ovarian morphology, in non-epileptic rats (Svalheim et al. 2007). An *in vitro* study on cell cultures from pig ovaries support these findings (Taubøll et al. 2006). A significant increase in basal testosterone secretion and a significant decrease in estradiol secretion were observed when a co-culture of theca and granulosa cells was exposed to LEV in high concentrations. This study is the first to demonstrate an effect of LEV on ovarian steroidogenesis. In the same experiment the cells were stimulated with gonadotropins and detected no effect of LEV on the hormone secretion was detected (Taubøll et al. 2006).

LEV has a mechanism of action which differs from all other AEDs. Lynch et al. have shown that the effect is from the drug binding to special vesicle proteins called SV2A (Lynch et al. 2004). This protein is found throughout the central nervous system (Bajjalieh et al 1994), but it has also been found in most endocrine tissue. Portela-Gomes and co-workers used a monoclonal antibody to SV2A to detect its occurrence in several parts of the human neuroendocrine system including the anterior pituitary gland, pancreas, adrenal medulla, thyroid, parathyroid, and neuroendocrine cells in the GI-tract (Portela-Gomes et al. 2000).

Due to these findings one might assume that there is a possibility that LEV may bind to SV2A in endocrine tissues, including the gonads and have a specific effect there in addition to its effect in the central nervous system.

Differential effects of antiepileptic drugs on hormone production in H295R, a human *in vitro* model for adrenal steroidogenesis

Aims of the study

The H295R cell-line has been shown to be a unique model system to study potential effects on steroidogenesis since it allows both the measurements of alterations in gene expression and in steroid hormone production in the same cell cultures. In the present study the principal objective was to study possible effects of AEDs on the steroidogenesis. Our drugs of interest were LEV, VPA and CBZ. The first approach was to investigate how AEDs affected hormone production and viability in the H295R cell line.

Rationale for choosing the H295R cell model

The H295R cell-line and has all the enzymes necessary to produce steroid hormones. The cells have the ability to produce each of the zone-specific steroid groups (Oskarsson et al. 2006). The genes for key-enzymes that are expressed include CYP11a (cholesterol side-chain cleavage), CYP11β2 (aldosterone synthetase), CYP17 (steroid 17α-hydroxylase and/or 17, 20 synthetase), CYP19 (aromatase), 17β-HSD1 and 17β-HSD4 (17β-hydroxysteroid dehydrogenase, type 1 and 2), CYP21β 2 (steroid 21-hydroxylase), 3β-HSD2 (3β-hydroxysteroid dehydrogenase), HMGR (hydroxymethylgutaryl CoA reductase) and StAR (steroid acute regulatory protein). The H295R cell-line is derived from the NCI-H295 pluripotent adrenocortical carcinoma cell line from a primary carcinoma that arose in a 48 year old woman (Gazdar et al. 1990). The H295R-cells have been found to have a good correlation of response with normal adult human adrenal cells (Oskarsson et al. 2006) and it has been found to be responsive to pharmaceuticals, including endocrine modulators (Gracia et al. 2007).

The adrenal gland is known to be the most common endocrine organ associated with chemically induced lesions. Within the adrenal gland, the zona fasciculata and the zona reticularis are most frequently affected (Ribelin et al. 1984). A number of factors predispose the adrenal cortex for direct toxic insults, such as its large blood supply pr unit mass, its lipophilicity and its high concentration of CYP 450 and other enzymes normally involved in

the steroidogenesis but with the potential capacity of bioactivate toxicants (Harvey et al. 2003). One important regulatory substance influencing on enzymes is the steroidogenetic factor 1 (SF-1) (Lala et al. 1992).

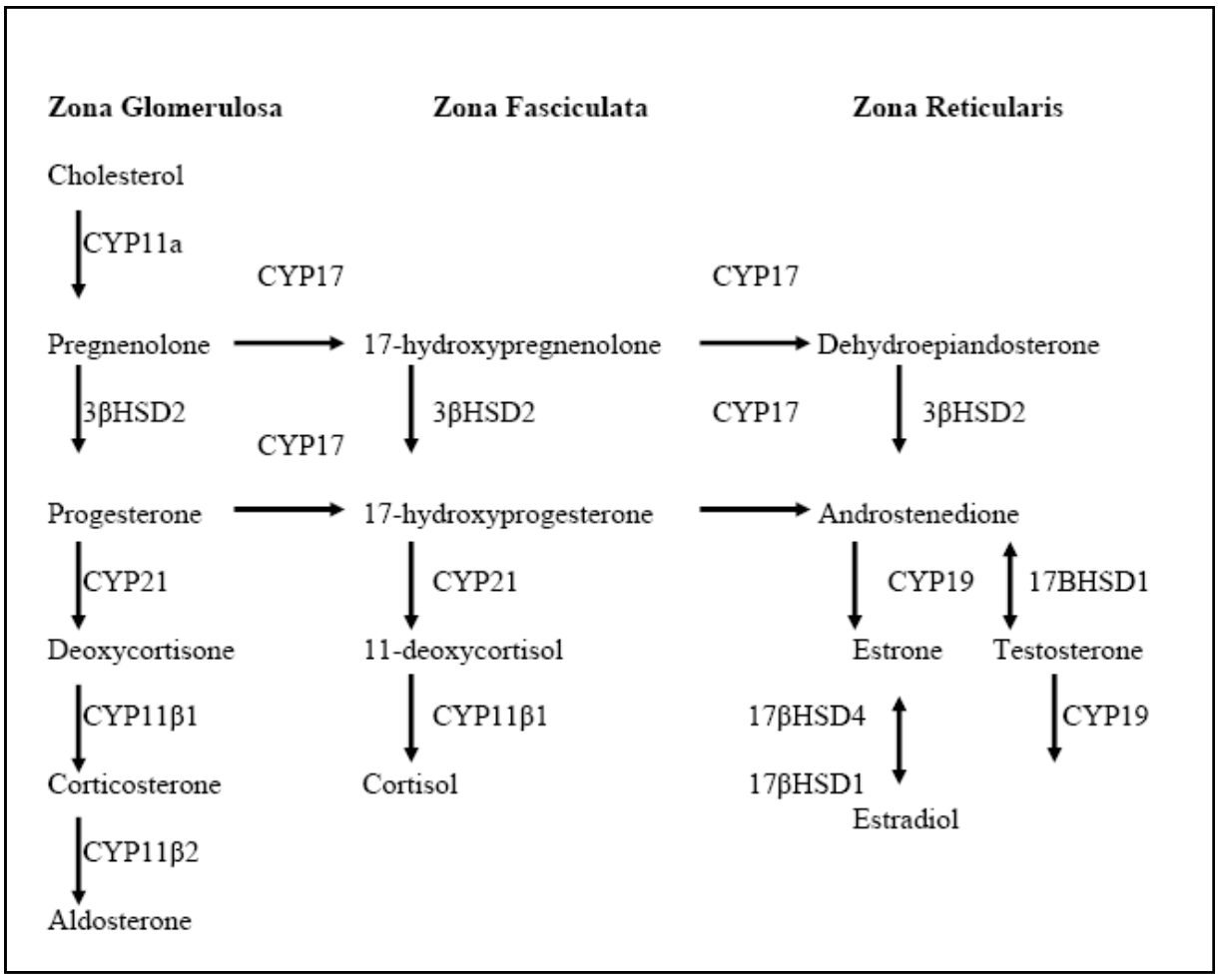

Figure 1: Steroidogenesis in the adrenal cortex

Materials and methods

Reagents

Valproic acid salt dissolved in medium to a 300 mM solution and stored -20°C. Levetiracetam from capsules dissolved in syrup (syrup saccharid 655 g, spiritus conservans 5 g and aqua purificata ad 1000 ml made by the Pharmacy at Rikshospitalet University Hospital, Oslo, Norway) to a 175 mM solution and stored at 4°C. Carbamazepine dissolved in EtOH to 100 mM solution and stored in room-temperature. Forskolin (Sigma-Aldrich)

dissolved in DMSO to 10 mM in and stored at -20°C. Prochloraz (Sigma-Aldrich) was dissolved in DMSO to 30 mM and stored at -20°C.

Cell-culture

The H295R-cells were obtained from American Type Culture Collection (ATCC # CRL-2128, Manassas, VA, USA) and cultured according to the protocol described by Gracia et al. 2006. In short, cells were grown in 75m² flasks with 12.5 ml of supplemented medium at 37 °C with 5% CO2 atmosphere. The medium was D-MEM/F-12(1x) liquid 1:1 with HEPES buffer, L-glutamine and pyridoxine HCl (Invitrogen). The medium was supplemented with 5 ml ITS+premix (BD Biosciences) and 12.5 ml NuSerum (BD Bioscience) to 500 ml medium. The medium was stored at 4 °C. Stock cultures of cells were given fresh medium every 2-3 days.

Exposure Design

The cells were detached from the flask using 0.25% trypsin-EDTA (Invitrogen) and split when they were approximately 90% confluent. Cells were counted with a Bürker haemacytometer. For exposures, cells were seeded in 1 ml medium at 300 000 cells/well in 24-well plates. Cells were incubated overnight. The medium was refreshed and the chemicals added. Concentrations used for CBZ were 18 µM, 37 µM, 55 µM, 67 µM, 83 µM and EtOH 0.1% was used as solvent control, for LEV 50 µM, 75 µM, 100 µM, 125 µM, 175 µM and syrup 0.1% was used as solvent control, for VPA 300 µM, 600 µM, 900µM, 1200 µM, 1500 µM and medium was used as solvent control. In addition, cells were exposed to control chemicals with known effects on steroidogenesis and related gene expression (Gracia et al. 2006). These included the cAMP-enhancer, forskolin (1 µM and 10 µM and DMSO 0.1% used as solvent control), and the imidiazol fungicide, prochloraz (3 µM and 0.3 µM and DMSO 0.1% used as solvent control) a known inhibitor of many CYP enzymes, including aromatase. Cells were exposed for 48 hr. In three independent repeats medium was collected for hormone quantization and stored at -75 °C until analysis. Medium was replaced, and 10% AlamarBlue (Invitrogen) added to assess cell viability. Cells were incubated 3 hr and a 100µl sample of each treatment was used to obtain the absorbance at 570 nm and 600 nm and the values were expressed as percentage to controls (Appendix).

Hormone analysis

Concentrations of hormones in media were measured with solid-phase radioimmunoassay/coated tube radioimmunoassay.

For detection of testosterone, estradiol, and cortisol concentrations Coat-A-Count® kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA) were used. The sensitivity of the testosterone assay was 0.14 nmol/L and the intra and interassay coefficients of variation were 5-20 % and 5.9-12 %, respectively. The sensitivity of estradiol assay was 8 pg/mL and the intra and the interassay coefficients were 4.0-7.0% and 4.2-8.1%, respectively. The sensitivity of cortisol assay was 5.5 nmol/L and the intra and interassay coefficients were 3.0-5.1% and 4.0-6.4%, respectively. For detection of progesterone levels we used Spectria Progesterone RIA kit (Orion Diagnostica, Espoo, Finland). The sensitivity of the progesterone assay was 0.3 nmol/L and the intra and interassay coefficients were 3.5-7.9 % and 3.7-5.6 %, respectively.

Hormone levels were measured twice in every sample and a mean concentration was calculated.

Statistical analysis

Concentrations of the individual hormones were compared to the levels seen in solvent-exposed cells and expressed as percentage of control.

Data were analyzed by JMP 7 software (SAS Institute Inc 2007). The observed values were tested for normality by using the Shapiro-Wilk's test (Shapiro and Wilk, 1965). Most data showed a distribution pattern indicating non-normality. Differences between mean values were tested using the Wilcoxon signed-rank test. In case of non-normality in the dependent variables a logarithmic transformation was performed to make a better fit to the normal distribution. General linear models (GLM) were used to assess dose-response relationships. Dependent variables were hormone concentrations relative to control. Independent variables were tissue culture experiment, exposure dose, and the interaction between experiment and exposure dose. P-values less than 0.05 were regarded to be statistically significant.

Results

Findings in VPA-exposed cells

The exposures of the cells with VPA caused a significant dose-dependent decrease in the production of estradiol (Fig 2 and 3). The estradiol levels were reduced to 40% of controls at a VPA concentration of 600 μM and to 30 % of control at a VPA concentration of 1500 μM. The testosterone to estradiol ratio for the different concentrations of VPA was calculated (Fig 4) and a dose-dependent increase was observed.

Moreover, we found a statistically significant reduction of both progesterone and cortisol levels in the VPA-exposed cells. For progesterone, the reduction was significant for all concentrations of VPA except the lowest, and for cortisol the reduction was significant for the concentrations of 300μM, 900μM and 1500μM.

For all the hormones investigated there was a significant dose-response relationship with increasing VPA exposure dose.

Findings in CBZ-exposed cells

For the cells exposed to CBZ we detected a statistically significant increase of estradiol levels for all the concentrations, except for 55.6 μM. We also found a statistically significant decrease of cortisol levels in the cells exposed to the two highest concentrations of CBZ (Fig. 5). A significant dose-response relationship was found on progesterone and cortisol concentrations, but no such effect was seen for estradiol or testosterone.

Findings in LEV-exposed cells

For the cells exposed to LEV, no dose-response relationship was seen (Fig. 6). At exposure dose 50 μM we found a significant reduction of estradiol and progesterone levels, and for the concentration of 125 μM a significant reduction of estradiol levels was detected.

Findings in the cells exposed to positive controls (The QC-plate)

Forskolin significantly increased the production of all hormones and the increase of estradiol and cortisol levels was found to be dose-dependent (Fig 7 and 8).

Prochloraz significantly increased progesterone levels in a dose-dependent manner and decreased levels of estradiol, testosterone and cortisol in a dose-dependent manner (Fig. 9).

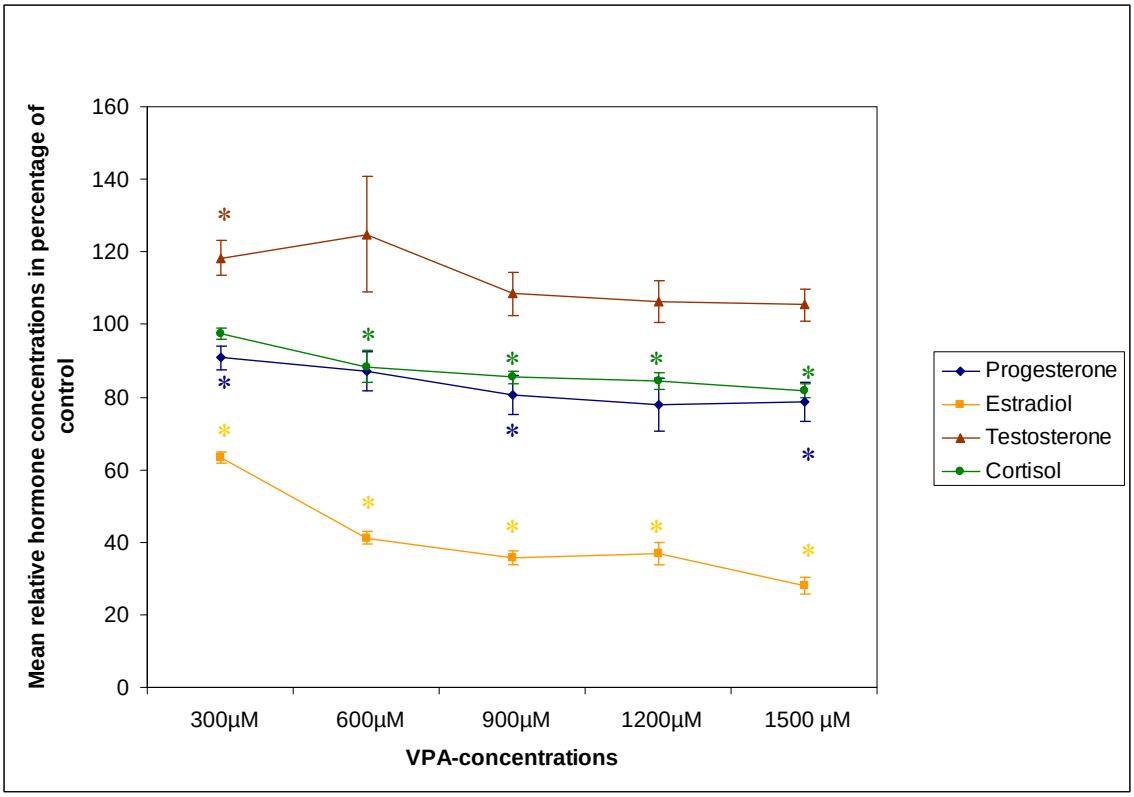

Figure 2: Mean (±SE) relative hormone concentrations (percent of unexposed controls) in H295R cells exposed to VPA in the dose range: 300 µM-1500 µM (* p< 0.05, Wilcoxon signed-rank test)

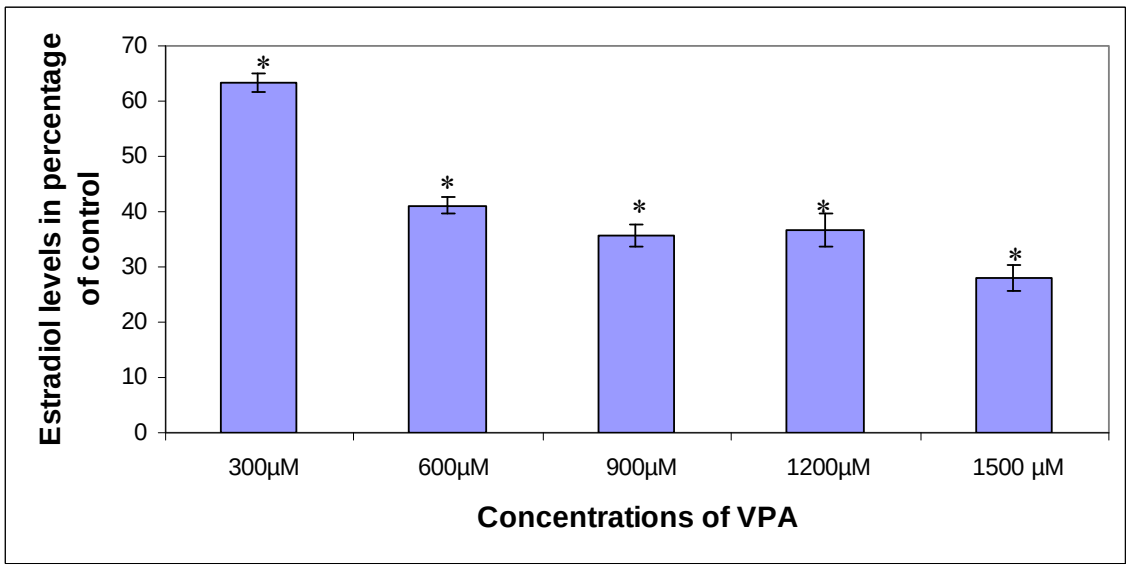

Figure 3: Mean (±SE) relative estradiol concentrations (percent of unexposed controls) in H295R cells exposed to VPA in the dose range: 300 µM-1500 µM (* p< 0.05, Wilcoxon signed-rank test)

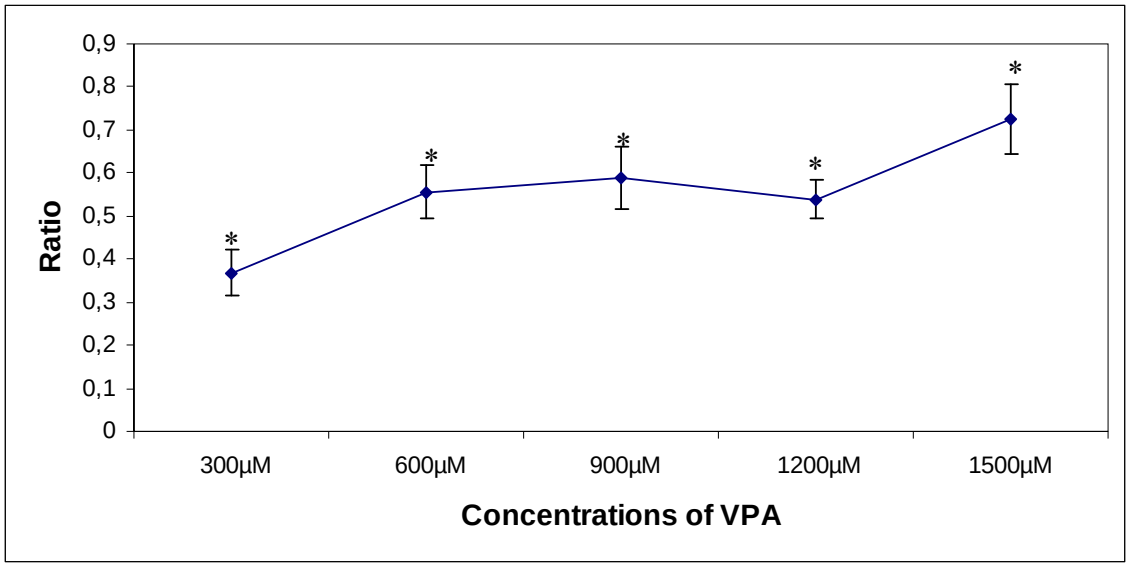

Figure 4: Ratio testosterone/estradiol in the cells exposed to valproate in the dose range: 300 µM-1500 µM (* p< 0.05, Wilcoxon signed-rank test)

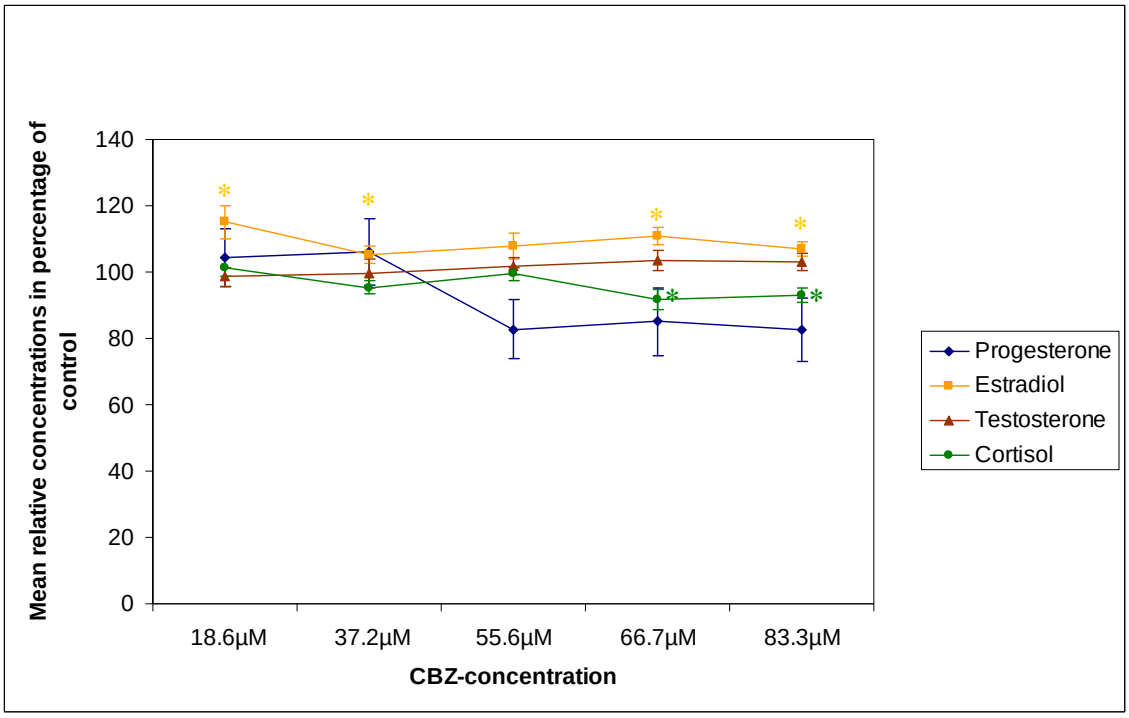

Figure 5: Mean (±SE) relative hormone concentrations (percent of unexposed controls) in H295R cells exposed to CBZ in the dose range: 18.6 µM-83.3 µM (* p< 0.05, Wilcoxon signed-rank test)

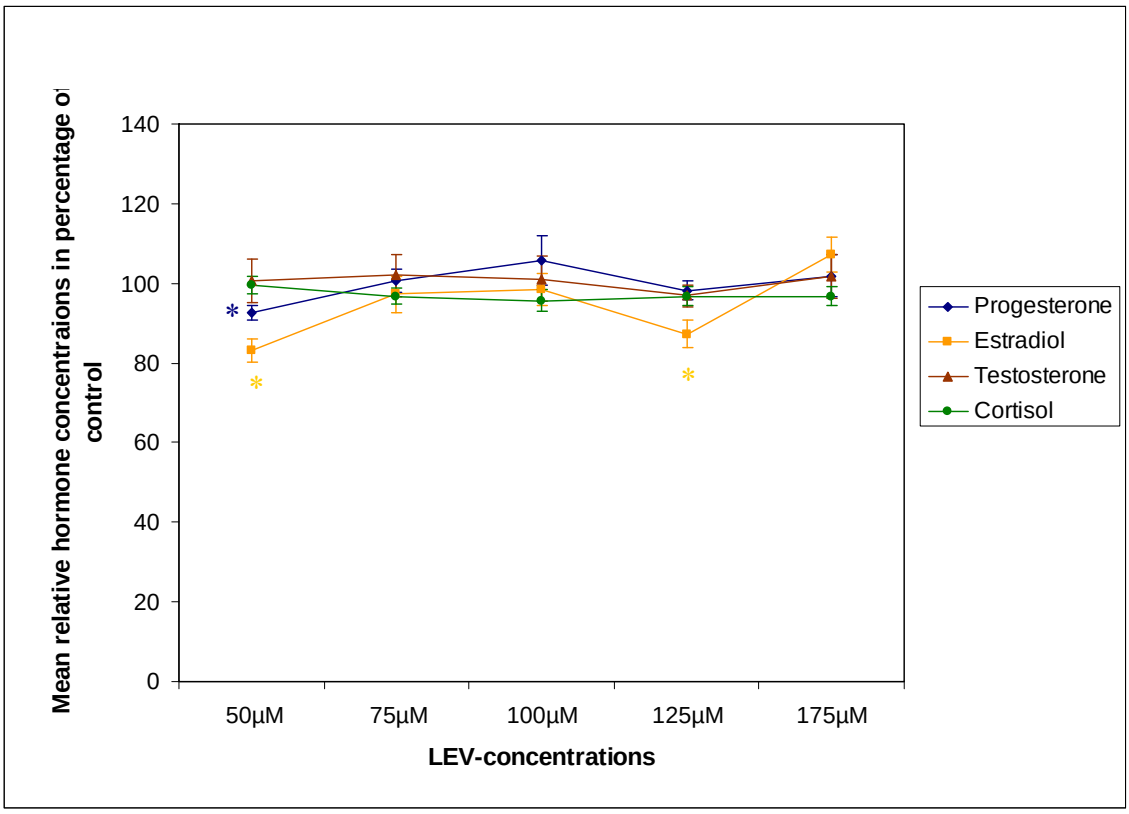

Figure 6: Mean (±SE) relative hormone concentrations (percent of unexposed controls) in H295R cells exposed to LEV in the dose range: 50 µM-175 µM (* p< 0.05, Wilcoxon signed-rank test)

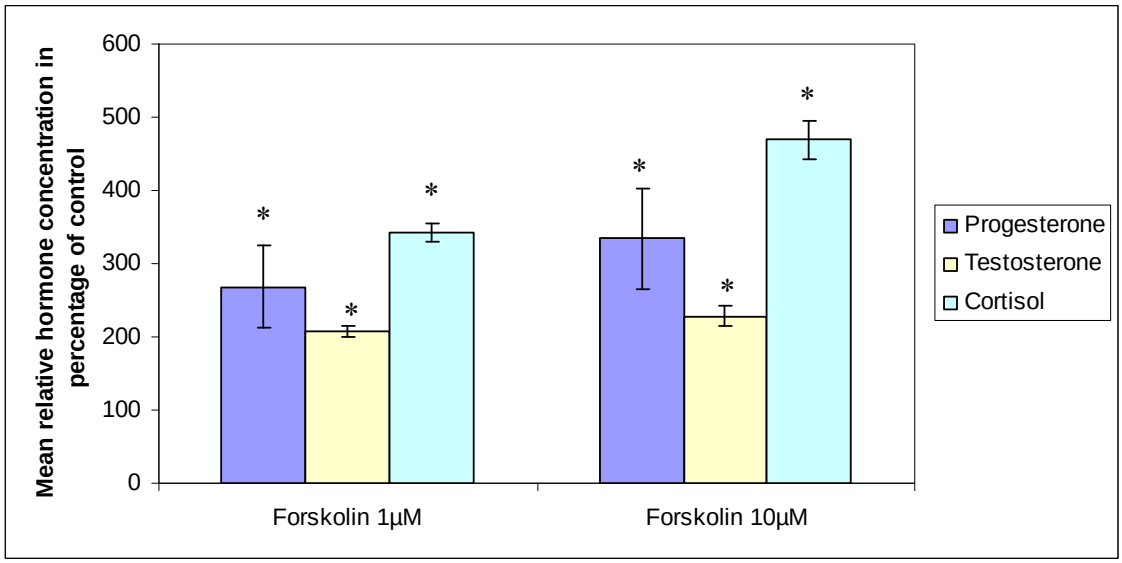

Figure 7: Mean (±SE) relative hormone concentrations (percent of unexposed controls) in H295R cells exposed to forskolin in the dose range: 1µM-10µM (* p< 0.05, Wilcoxon signed-rank test)

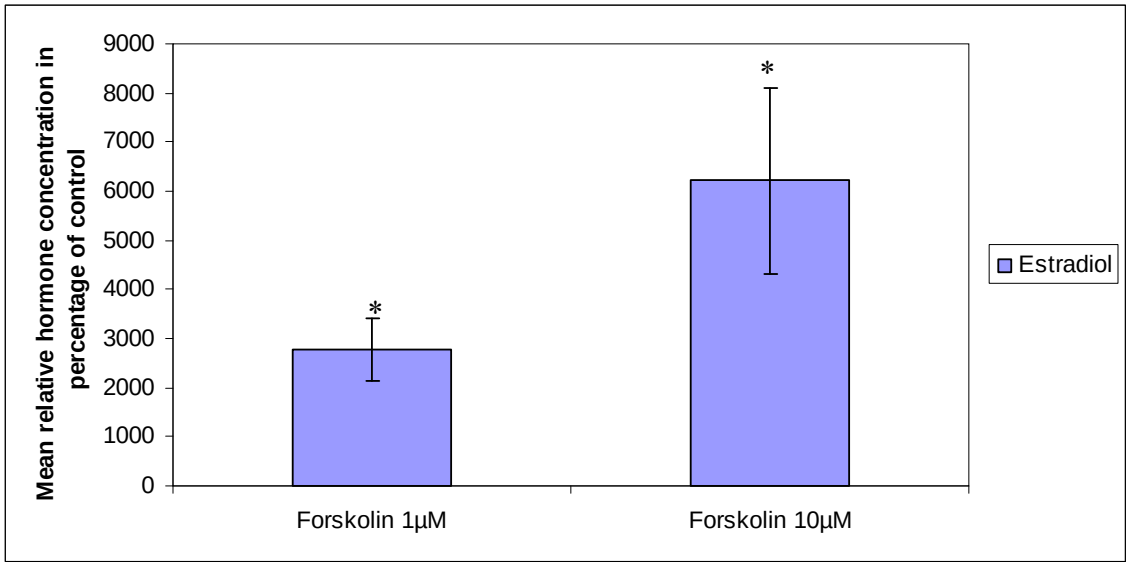

Figure 8: Mean (±SE) relative estradiol concentration (percent of unexposed controls) in H295R cells exposed to forskolin in the dose range: 1µM-10µM (* p< 0.05, Wilcoxon signed-rank test)

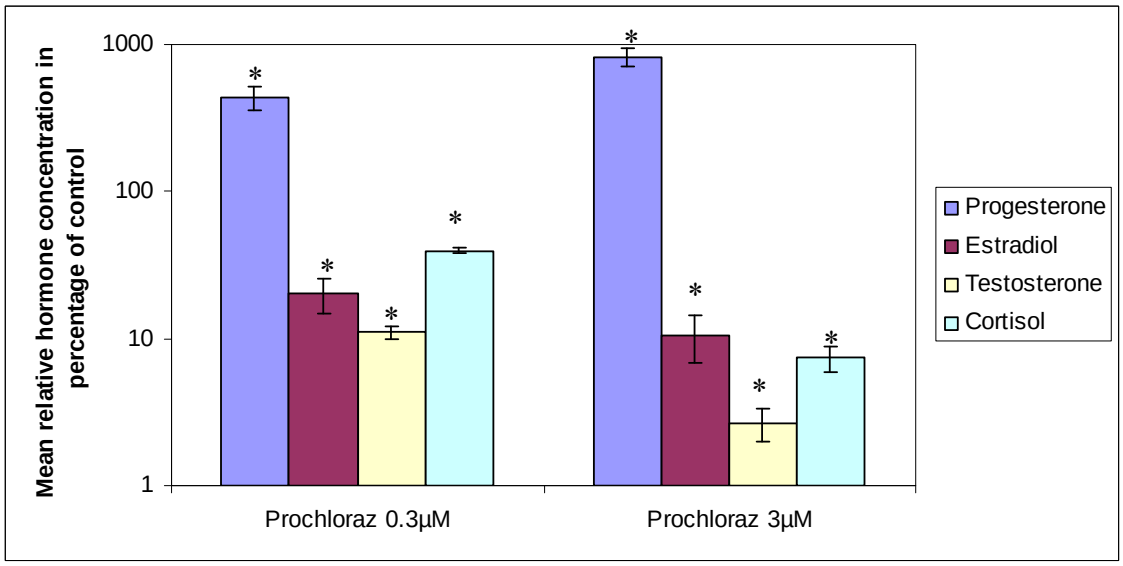

Figure 9: Mean (±SE) relative estradiol concentration (percent of unexposed controls) in H295R cells exposed to prochloraz in the dose range: 0.3µM-3µM (* p< 0.05, Wilcoxon signed-rank test)

Discussion

The cells exposed to the VPA, CBZ and LEV showed different patterns of hormone production.

In the cells exposed to VPA we detected a significant dose-dependent decrease of the estradiol levels. The effects were significant even within the therapeutic range, thus can be considered relevant for humans. The alterations showed a linear, dose-response relationship indicating that higher dose levels were associated with a stronger endocrine disrupting effect. Moreover, testosterone levels in the VPA-exposed cells were found to be significantly increased for the lowest concentration of VPA. We also detected a significant decrease in the progesterone levels for three of the VPA-concentration.

The most prominent finding in the VPA-exposed cells was a significant dose-dependent increase of the testosterone to estradiol ratio, mainly due to a decrease in the estradiol secretion. This finding supports previous *in vitro* findings in cell-models such as porcine ovarian follicular cells and *in vivo* findings both in animal studies and in clinical studies. Gregoraszczuk et al. (2000) reported a direct effect of VPA on the steroidogenesis in porcine follicular cells. They found a significant and dose-dependent reduction both in testosterone and estradiol secretion from follicular cells, but the testosterone to estradiol ratio was significantly increased indicating that VPA inhibits the conversion of testosterone to estradiol. They also found a reduction of progesterone levels in the cells exposed to VPA. In the same cell-model, it has also been detected that the effect of VPA on the steroidogenesis is irreversible. In this study a significant reduction of estradiol secretion and an increase in both testosterone and progesterone secretion was detected (Taubøll et al. 2002). These findings stand in contrast to the finding in the present data of a significant decrease in progesterone levels. This apparent discrepancy could be due to the fact that two different cell-models were used and that the effects of VPA on ovarian steroidogenesis could differ from the effects on the adrenal steroidogenesis.

Taubøll and co-workers have also reported that VPA inhibit the conversion of testosterone to estradiol in gonadotropin-stimulated and in non-stimulated porcine ovarian follicular cells (Taubøll et al. 2003, 2006). Moreover, several *in vivo* findings support an increased testosterone to estradiol ratio caused by VPA. Røste et al. (2002) have revealed an increased testosterone to estradiol ratio in both male and female non-epileptic rats after they

were fed with VPA in therapeutic dose. Moreover, a large number of clinical studies have detected an elevated testosterone level in women treated with VPA (Isojärvi et al. 1993, 1996, 2001, Murialdo et al. 1998). In addition, this suggestion is supported by the finding of an inhibition of VPA on aromatase complex (CYP 19) in microsomes from insect cells expressing human recombinant CYP 19 (Jacobsen et al. 2008).

Another mechanism of action that has been suggested is that VPA might inhibit the enzyme mEH (Kerr et al 1989, Bernus et al. 1997) and it has been demonstrated that the inhibition of this enzyme suppresses the conversion of testosterone to estradiol in human granulosa cells (Hattori et al. 2000). In what manner this mechanism is relevant for our findings in the H295R-cells is difficult to determine without the knowledge of the occurrence of this enzyme in the cells. mEH has been detected in high levels in the human adrenal cortex (Papadopoulos et al. 1992), but some investigations indicate that this enzyme has no effect on the adrenal steroidogenesis and its function here is still unclear (Papadopoulos et al. 1994).

Moreover, VPA is known to be a HDAC inhibitor and an *in vitro* study suggests that VPA diminish steroid hormone production by modulating the transcription factor SF-1 and thereby suppressing the early steroidogenetic gene CYP11A1 (Chen et al. 2007).

The cells exposed to LEV did not show any consistent biological variation of hormone production. In the cells exposed to 50 μM we found a significant reduction of estradiol and progesterone levels and in the cells exposed to 125 μM a significant reduction of estradiol levels was detected. No dose-response relationship was observed.

It has not been reported any endocrine side-effects of LEV in humans up to date. However, Taubøll et al. (2006) have showed that LEV affected basal steroid hormone production in ovarian follicular cells from prepubertal pigs. Moreover, LEV has been found to affect the hormonal balance in female non-epileptic rats by increasing serum testosterone levels, reducing estradiol levels, progesterone levels and FSH levels (Svalheim et al. 2008). In addition, the treated animals had a larger number of corpora lutea and secondary follicles in the ovaries than the control animals.

The mechanism by which LEV might alter the reproductive endocrine balance is unknown, but LEV is known to bind to the synaptic vesicle protein SV2A (Lynch et al. 2004). This protein is found throughout the central nervous system (Bajjalieh et al 1994) and in addition in most endocrine tissue. The SV2A has been detected in the adrenal medulla, but not in the adrenal cortex (Portela-Gomes et al. 2000). This might be one explanation why no consistent alterations occurred in our experiment.

We found a significant increase of estradiol levels in the cells exposed to CBZ for all concentrations except 55.6 μM. The increase was dose-dependent in a significant manner, but the interaction test indicated that only a small part of the variation could be attributed to the tested variables. Moreover, the data suggested a significant dose-dependent decrease of cortisol production in cells exposed to the two highest concentrations of CBZ.

CBZ has a well-known effect on the endocrine system *in vivo* by induction of the hepatic CYP 450 enzyme system. This results in an increase in the metabolism of some hormones and the synthesis of SHBG, which again leads to decreased levels of free fractions of sex-hormones. The fact that the H295R cell-model only reflects direct cell-effects may explain differences in responses compared with *in vivo* responses. Thus, the findings in the cells exposed to CBZ are probably due to other mechanisms.

Our findings in the quality-control plate, consisting of cells exposed to forskolin and prochloraz, are in accordance to previous findings and this indicates a high reliability of the cell-model (Gracia et al. 2006, Hecker et al. 2006).

The viability of the cells exposed to the different drugs is needed to be kept in mind when the findings are evaluated. The viability was tested with AlamarBlue. The cells exposed to LEV and CBZ showed stabile viability (Appendix), but in the cells exposed to VPA we found a tendency towards reduced viability in the highest concentrations (Appendix). It has been suggested that VPA can act as an apoptotic agent by increasing caspase-3 activity in a dose-dependent manner (Taubøll et al. 2003) and this might be an explanation for the reduction. Moreover, the reduction in estrogen levels in the VPA-exposed cells could also explain the reduction in AlamarBlue conversion seen at the highest doses under these conditions. Indeed, it has been suggested H295R cell proliferation is dependent on estradiol (Montanaro et al. 2005). Thus, reduced levels of estradiol would inhibit H295R proliferation, and thus subsequent AlamarBlue conversion.

Further research is required to elucidate our findings and detect possible mechanisms of action of the AEDs on the steroidogenesis. We have now started to investigate the levels of gene expression for several steroidogenetic enzymes in the H295R cells, and these results will hopefully contribute to further understanding. Further experiments looking at the expression of genes such as the nuclear receptors and steroidogenetic regulation factors SF-1 and DAX-1 might give a better understanding of the mechanisms regulating steroidogenesis and the possible effect of AEDs on these factors and then again on the hormone production.

Moreover, stimulation of the H295R cells with e.g. ACTH might be one way to create a more clinically relevant situation. The H295R cells have been shown to both have

functional ACTH-receptors (Li and Wang 2005) and functional CRH-receptors (Willenberg et al. 2005). Oskarsson et al. have investigated the effect of forskolin on the cortisol production in the H295R cells and concluded that this made the cells more similar to the profile in the adult adrenal gland (Oskarsson et al. 2006).

Further research in other cell-models is also to be encouraged, e.g. in cell-models from theca and/or granulosa cells from the ovaries or Leydig cells from the testicles. Investigations of the effect of AEDs in the hypothalamus and the pituitary might also lead to a better understanding of the possible mechanisms of action on the hormonal balance.

Conclusions

Our finding of an increased testosterone to estradiol ratio, mainly due to a decrease in the estradiol production in the cells exposed to VPA is consistent with previous findings both *in vitro* and *in vivo*. This indicates that VPA inhibits the conversion of testosterone to estradiol. The lack of prominent alterations of hormone levels in the cells exposed to LEV and CBZ might be explained by the fact we utilized an *in vitro*-model for investigation of direct cell-effect. This emphasizes the need for more studies, e.g. with stimulated H295R-cells, in other cell-models, in animal models and in humans.

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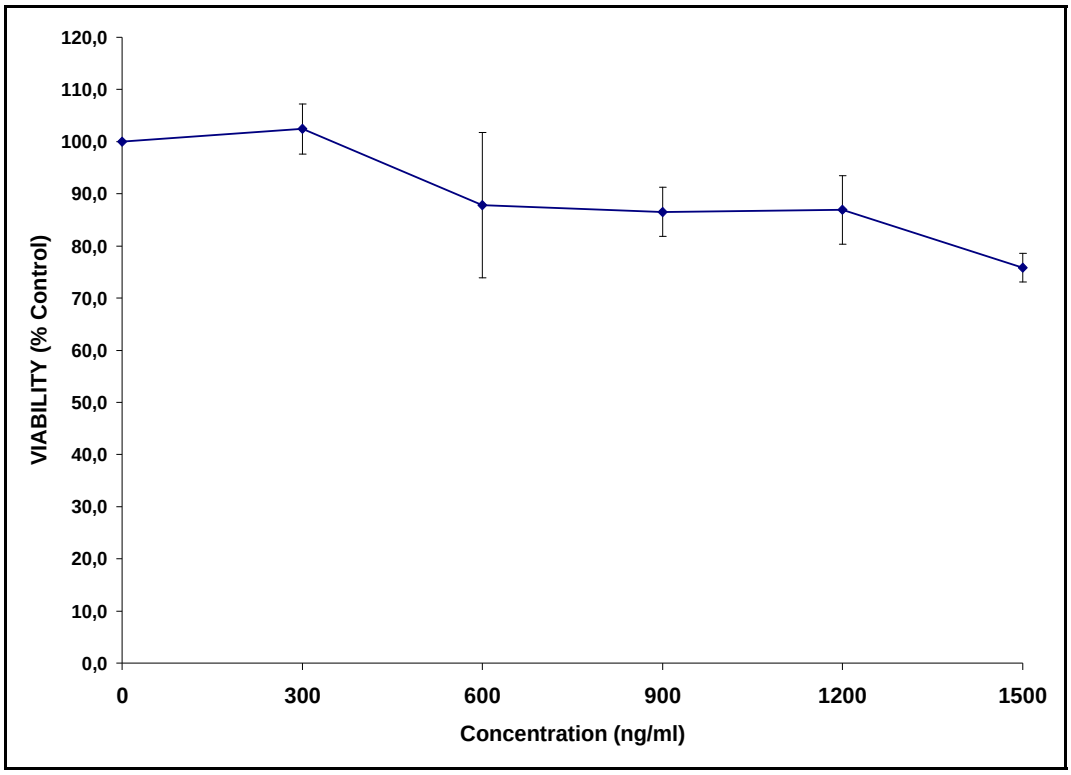
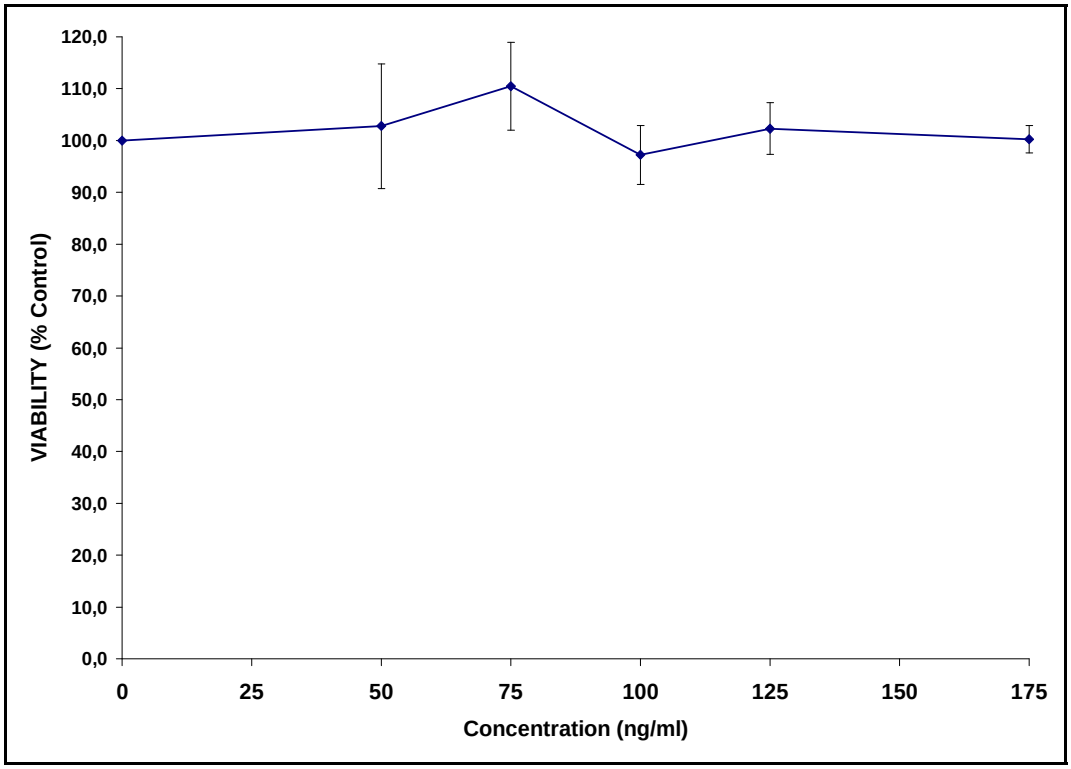
Appendix

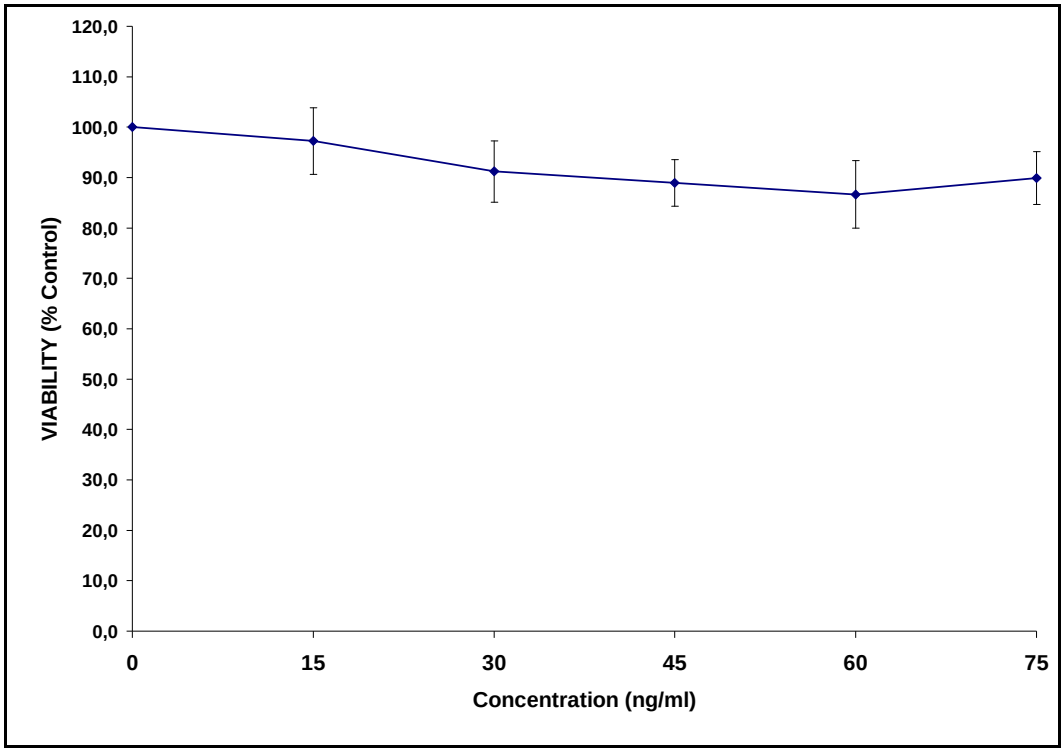
AlamarBlue testing of cells exposed to VPA



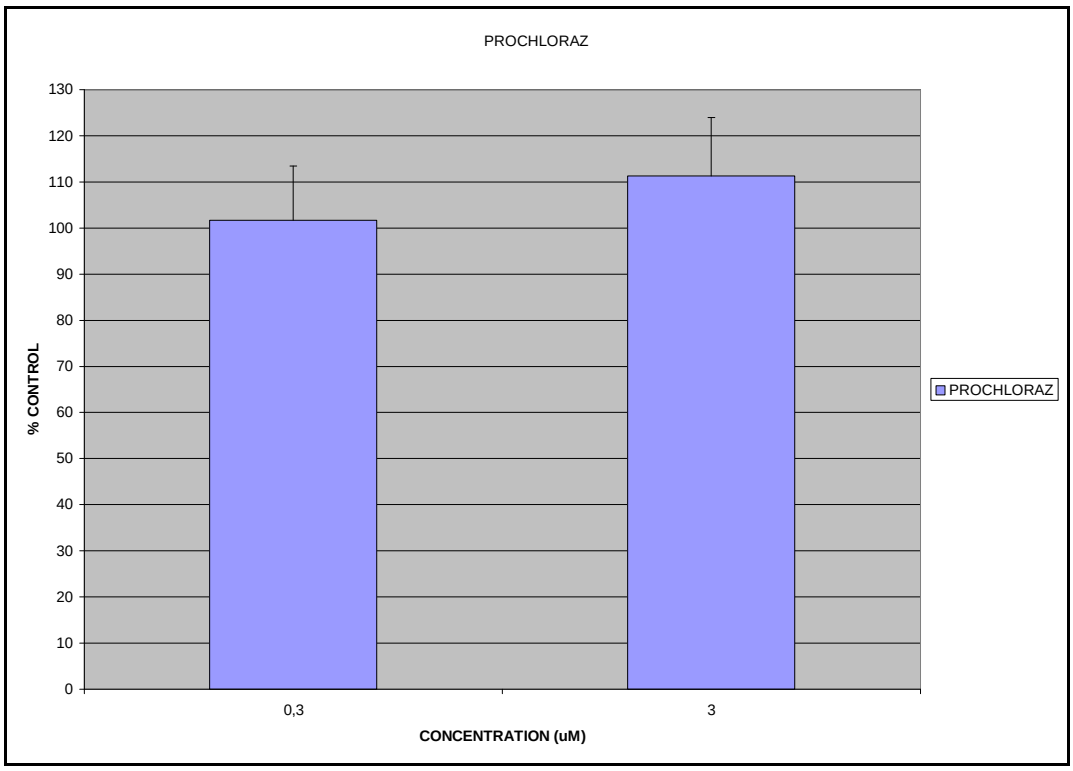
AlamarBlue testing of cells exposed to LEV



AlamarBlue testing of cells exposed to CBZ



AlamarBlue testing for cells exposed to prochloraz



AlamarBlue testing of cells exposed to forskolin