J. Steroid Biochem. Molec. Biol. Vol. 43, No. 7, pp. 641–648, 1992
Printed in Great Britain

0960-0760/92 $5.00 + 0.00
Pergamon Press Ltd

# EVALUATION OF THE RACEMATE AND THE ENANTIOMERS OF A NEW HIGHLY ACTIVE AND SELECTIVE AROMATASE INHIBITOR OF THE AMINOGLUTETHIMIDE TYPE

R. W. HARTMANN,* G. GRÜN, U. BARTZ and M. PALZER
Fachrichtung 12.1 Pharmazeutische Chemie, Universität des Saarlandes, 6600 Saarbrücken, Germany

*(Received 15 June 1992)*

**Summary**—Compound 1 [3-(4-aminophenyl)-3-cyclohexylpiperidine-2,6-dione] is a highly potent nonsteroidal aromatase inhibitor of the aminoglutethimide (AG)-type containing an asymmetric carbon atom. 1 and its enantiomers (+)-1 and (−)-1 inhibited human placental aromatase by 50% at 0.3, 0.15, and 4.6 $\mu$M, respectively ($IC_{50}$ AG = 37 $\mu$M). A competitive type of inhibition was observed for 1 and (+)-1 ($K_i$ 1 = 3.9 nM, $K_i$ (+)-1 = 2.0 nM, $K_i$ AG = 408 nM). Using solubilized high spin aromatase, 1 showed a type II difference spectrum indicating the interaction of the amino nitrogen with the central Fe(III)-ion of the cytochrome $P_{450}$ heme component. 1 and (+)-1 inhibited cholesterol side chain cleavage enzyme (desmolase) by 50% at 67 and 82 $\mu$M, respectively ($IC_{50}$ AG = 29 $\mu$M). In ACTH-stimulated rat adrenal tissue *in vitro*, 1 was less active in inhibiting aldosterone and corticosterone production compared to AG ($IC_{50}$s, 1, 130 and 140 $\mu$M, AG, 80 and 50 $\mu$M, respectively). *In vivo*, 1 was superior to AG, too: it showed a stronger inhibition of the plasma estradiol concentration of pregnant mares' serum gonadotropin-primed SD rats, the activity residing mainly in the (+)-enantiomer [ovarian vein: (+)-1, 0.31 mg/kg: 81% inhibition, (−)-1, 0.31 mg/kg: 6%, AG, 1.25 mg/kg: 35%]. Furthermore 1 was much more active in inhibiting the testosterone-stimulated tumor growth of the ovariectomized 9,10-dimethyl-1,2-benzanthracene tumor-bearing SD rat (postmenopausal model). Up to a dose of 600 mg/kg of 1 no central nervous symptom depressive effects were observed in the motility test and the rotarod experiment, whereas AG exhibited $ED_{50}$s of 62 and 164 mg/kg, respectively.

## INTRODUCTION

Inhibition of the enzyme aromatase has been identified as a rational strategy for the therapy of estrogen-dependent diseases, e.g. estrogen-dependent breast cancer. Aromatase is a cytochrome $P_{450}$-dependent enzyme catalyzing the last step of estrogen biosynthesis [1]. A strong and selective inhibition of aromatase leads to an inhibition of estrogen formation without affecting the biosynthesis of other steroid hormones. So far the only commercially available aromatase inhibitor is aminoglutethimide (AG, Fig. 1). It has been effectively used in the treatment of estrogen-dependent breast cancer in postmenopausal women [2, 3]. The compound is not a selective aromatase inhibitor, however, for it also inhibits other cytochrome $P_{450}$-dependent enzymes involved in steroidogenesis at concentrations similar to those at which it inhibits aromatase [4, 5]. Furthermore AG also shows moderate tolerability in that it exhibits central nervous system (CNS) depressive activity leading to severe side effects like ataxia, somnolence and depression [6]. Thus the search was initiated for more active and more selective nonsteroidal aromatase inhibitors. Numerous attempts to optimize AG have been undertaken [7–13]. Investigations of our group showed that elongation of the ethyl substituent of AG leads to a strong increase in aromatase inhibition [14]. In a recent study we could demonstrate that the exchange of the 3-ethyl group of AG by a 3-cycloalkyl substituent (compound 1, Fig. 1) enhances aromatase inhibition even more dramatically [15]. We further showed that almost all of the aromatase inhibitory activity resides in the (+)-enantiomer [15], a finding which had been observed with AG as well [16].

This article describes the effects of the racemate and the enantiomers of compound 1 and AG on human placental aromatase and bovine adrenal desmolase, the inhibition of the

*To whom correspondence should be addressed.

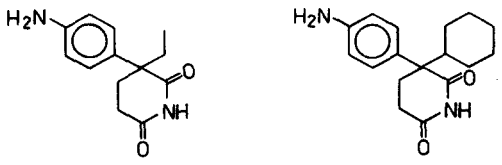


Fig. 1. Chemical structures of AG and compound 1.

aldosterone and corticosterone biosynthesis *in vitro*, the reduction of the plasma estradiol concentration, the mammary tumor inhibiting activity, and the activity in the rotarod experiment and the motility test *in vivo*.

## MATERIALS AND METHODS

### *Chemicals*

[1β,2β-$^3$H]testosterone (sp. act. 53.5 Ci/mmol) was obtained from New England Nuclear (Dreieich, Germany). Glucose-6-phosphate dehydrogenase (350 EU/mg) and $ACTH_{1-24}$ were purchased from Serva (Heidelberg, Germany). DMBA, pregnant mares' serum gonadotropin (PMSG) (1500–4000 IU/mg) and NADPH were obtained from Sigma (Munich, Germany). For the determination of the estradiol, corticosterone, and aldosterone concentration the radioimmunoassay (RIA) kits of DRG Instruments (Marburg, Germany) were used. CGS 16 949 A (4-(5,6,7,8-tetrahydroimidazo[1,5-*a*]-pyridin-5-yl)benzonitrile monohydrochloride) was a gift from Ciba Geigy (Basel, Switzerland), R 76 713 (6[(4-chlorophenyl)(1*H*-1,2,4-triazol-1-yl)methyl]-1-methyl-1*H*-benzotriazole) a gift from Janssen (Beerse, Belgium).

### *Animals*

Female SD rats and NMRI mice were obtained from Zentralinstitut für Versuchstierzucht (Hannover, Germany), the male SD rats from Savo (Kisslegg, Germany). The animals were fed with a commercial solid diet and tap water *ad libitum*. They were housed at room temperature (22°C) on a 12 h light–darkness cycle.

### *Preparation of aromatase and desmolase*

Aromatase was obtained from the microsomal fraction of freshly delivered human term placenta according to the method of Thompson and Siiteri [17]. The microsomes were resuspended in a minimum volume of phosphate buffer (0.05 M, pH = 7.4) and stored at −30°C. No loss of activity was observed within 4 months. Preparation of mitochondrial desmolase was carried out as described by Hochberg *et al.* [18] using bovine adrenal cortex as source. After resuspension in buffered (0.01 M Tris, pH = 7.4) sucrose (0.25 M) the mitochondria were stored at −70°C. Enzyme activity was stable within 3 months. Protein concentrations were measured according to Lowry *et al.* [19].

### *Aromatase inhibition in vitro*

Enzyme activity was monitored using the tritiated water method of Thompson and Siiteri [17]. The incubations were performed as described previously [14]. The $^3H_2O$ formed during the conversion of [1β,2β-$^3$H]testosterone to estradiol was determined after separation of the steroids by dextran-coated charcoal (DCC). Following centrifugation the radioactivity of a 200 μl supernatant aliquot was determined. For the determination of the $IC_{50}$ values, compounds were tested in six appropriate concentrations. Plotting the percent inhibition vs the concentration of inhibitor on a semilog plot the molar concentration causing 50% inhibition was determined.

The $K_i$ values were determined using the same procedure with following modifications: concentrations of [$^3$H]testosterone 0.05–0.4 μM, microsomal protein: 20–40 μg/incubation, 1 mM NADPH and NADPH regenerating system (glucose-6-phosphate 10 mM and glucose-6-phosphate dehydrogenase 1 EU). The incubations (0.25 ml) with and without inhibitor were carried out for 15 min at 30°C under initial velocity conditions. The $K_i$ values were calculated from the Lineweaver–Burk [20] plots. The graphs were fitted by linear regression analysis.

The test on irreversible inhibition of aromatase was performed as described [14], by incubating microsomal aromatase with NADPH and inhibitor (0–50 μM) for 15, 30, and 60 min. After separation of the inhibitor by the addition of DCC, aromatase activity in the supernatant was measured as reported previously [14].

### *Difference spectra*

Partial purification and solubilization of human placental microsomes was performed as described [21, 22]. The ammonium sulfate precipitate was dissolved in phosphate buffer (0.05 M, pH = 7.4) containing EDTA 1 mM, dithiothreitol 0.1 mM, sodium cholate 0.05% and was stored at −70°C. After thawing, testosterone was added to the protein solution to

produce the high spin state. Inhibitor dissolved in ethanol was added to the sample cuvette and the difference spectrum was scanned from 350–500 nm at 20°C.

*Desmolase inhibition*

The method of Hochberg *et al.* [18] was applied with modifications [14]. Adrenal mitochondria were incubated with the substrate [26-$^{14}$C]cholesterol. Separation of the isocaproic acid from the substrate was performed by chromatography on alumina mini columns. Aliquots of the eluate were counted. Inhibition values are the mean of at least three experiments at an inhibitor concentration of 25 $\mu$M. For the determination of the $IC_{50}$-values the same procedure as described under *aromatase inhibition* was applied.

*Inhibition of corticosterone and aldosterone formation in vitro*

The procedure described by Häusler *et al.* [5] was used. Adrenals removed from male SD rats were cut into eight pieces each and transferred into tubes (eight pieces per tube, randomized) containing 2 ml of incubation medium (Krebs–Ringer bicarbonate, 8.4 mM glucose, saturated with $O_2$–$CO_2$ (95:5); pH 7.6 [23]). After preincubation (1 h, 37°C, shaking water bath), the medium was replaced by fresh medium containing $ACTH_{1-24}$ (0.1 $\mu$g/mg) with and without test compound. After a 2-h incubation, supernatant aliquots were taken. The determination of the corticosterone and aldosterone concentration was performed without extraction using the corresponding RIA kits. The $IC_{50}$-values were determined by plotting the percent inhibition vs the inhibitor concentration on a semilog plot.

*Effect on plasma estradiol ($E_2$) level in vivo*

Eight- to twelve-week-old female SD rats were primed with PMSG similar to the method of Brodie *et al.* [24]. Subcutaneous (s.c.) injections of 100 IU of PMSG in 500 $\mu$l of saline were applied every other day for 7 days. On day 8 the animals received a single s.c. injection of olive oil or of an olive oil solution of the test compound. After 6 h the rats were anesthetized and blood was taken from the ovarian vein and the heart. The plasma $E_2$ level was determined by RIA.

*Mammary tumor studies*

The tumor inhibiting effect was determined by using the 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced hormone-dependent mammary adenocarcinoma of the SD rat in the postmenopausal arrangement [14, 25, 26]. The methods applied for tumor induction and assignment to treatment groups have been described previously [27]. Animals bearing at least one tumor greater than 140 mm$^2$ were classified in groups of ten. One day before the start of the treatment, the ovaries of the tumor bearing rats were removed through incisions in the lumbar region of the back. Compounds were dissolved in olive oil and applied s.c. 6 times a week. The therapy was continued for 28 days. Measurement of tumor size and determination of body weight were made weekly.

*Motility test and rotarod experiment*

In both experiments female NMRI mice (20–25 g) were used. The test compounds were applied perorally in doses up to 600 mg/kg. The motility test was performed according to [28] measuring the locomotor activity of single animals over two total periods of activity. Each dose group consisted of 8 animals. In the rotarod experiment the procedure of Gross *et al.* [29] was applied. Each dose group consisted of 10 mice.

## RESULTS

*Aromatase inhibition*

The inhibition values of the test compounds toward human placental aromatase are given in Table 1. Exchange of the ethyl substituent of AG by a cyclohexyl group increased inhibitory potency by a factor of 123 (compound **1**). Showing a relatively potency of 240 the dextrorotary enantiomer of **1**, (+)-**1**, is 30 times as active as its antipode [(−)-**1**]. The same phenomenon is observed with AG, (+)-AG exhibiting a relative potency of 1.9, whereas (−)-AG is almost inactive (relatively potency: 0.1).

Table 1. Inhibition of human placental aromatase by the racemates and the enantiomers of 1 and AG

| Compound | $IC_{50}$ ($\mu$M)[a] | RP[b] | $K_i$ (nM)[c] |
|---|---|---|---|
| 1 | 0.3 | 123 | 3.9 |
| (+)-1 | 0.15 | 240 | 2.0 |
| (−)-1 | 4.6 | 8 | ND |
| AG | 37 | 1 | 408 |
| (+)-AG | 19 | 1.9 | ND |
| (−)-AG | 370 | 0.1 | ND |

[a] Concentration of testosterone used 5 $\mu$M. Mean values of at least six experiments. The deviations were within ±10%. [b] Relative potency, calculated from the $IC_{50}$ values and related to AG. [c] $K_m$ (testosterone): 46 ± 8 nM. ND, not determined.

In the test for irreversible aromatase inhibition [30] 1 and AG were inactive, i.e. no reduction of enzymatic activity was observed after preincubation for 1 h and subsequent removal of the inhibitor by DCC treatment (data not shown).

To get a further insight into the mode of aromatase inhibition, Lineweaver–Burk and difference spectroscopy experiments were performed with compound 1 and AG. Compound 1, (+)-1 and AG showed a competitive inhibition vs testosterone (figures not shown).

Based on the $K_i$ values, compound 1 is 105 times, (+)-1 is 204 times as potent an inhibitor of aromatase as AG ($K_i$ 1 = 3.9 nM, $K_i$ (+)-1 = 2.0 nM, $K_i$ AG = 408 nM, Table 1).

The difference spectrum of compound 1 toward solubilized high spin aromatase is shown in Fig. 2. Exhibiting a minimum at 390 nm and a maximum at 425 nm, the spectrum is typical of the interaction of a basic nitrogen (amino group of the $p$-$NH_2$ phenyl moiety) with the central Fe(III)ion of the cytochrome $P_{450}$ heme component (type II difference spectrum) [31]. The same type of difference spectrum was obtained with AG (data not shown).

The data obtained in the Lineweaver–Burk and spectroscopy experiments show that compound 1 interacts with the central iron of the cytochrome $P_{450}$ as well as the apoprotein moiety of the enzyme.

*Selectivity of inhibition*

As already pointed out, AG is a less selective aromatase inhibitor, as it also inhibits other cytochrome $P_{450}$-dependent hydroxylases of steroid biosynthesis leading to a depletion of mineralo- and glucocorticoid levels. In order to exclude those effects, new aromatase inhibitors have to be tested for selectivity of aromatase inhibition.

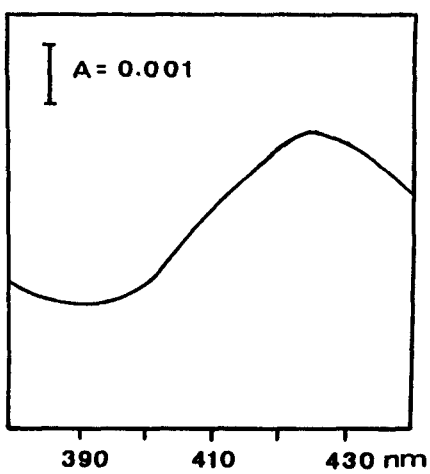


Fig. 2. Difference spectrum obtained by the addition of compound 1 to solubilized high spin aromatase. Both cuvettes contained: testosterone 5 μM, microsomal protein 0.96 mg/ml, ethanol 0.1%; only sample cuvette: compound 1 1 μM.

Table 2. Inhibition of bovine adrenal desmolase by the racemates and the enantiomers of 1 and AG

| Compound | % Inhibn[a,b] | $IC_{50}$ (μM)[b] | RP[c] |
|---|---|---|---|
| 1 | 31 | 67 | 0.4 |
| (+)-1 | 19 | 82 | 0.3 |
| (−)-1 | 39 | ND | — |
| AG | 57 | 29 | 1 |
| (+)-AG | 67 | ND | — |
| (−)-AG | 36 | ND | — |

[a]Concentration of inhibitor: 25 μM. [b]Mean values of at least 4 experiments. The deviations were within ±10%. [c]Relative potency, calculated from the $IC_{50}$ values and related to AG. ND, not determined.

Table 2 shows the effects of AG and compound 1 on the cholesterol side chain cleavage enzyme (desmolase), the key enzyme, which catalyses the first and rate-limiting step of steroid biosynthesis. Compared to AG the inhibitory activity of compound 1 toward desmolase is reduced (relative potency 0.4). (+)-AG shows a stronger inhibitory effect than the racemic mixture, whereas the opposite is true for compound 1. The most active aromatase inhibitor, compound (+)-1, is the least active inhibitor of desmolase.

The effects of compound 1 and AG on corticosterone and aldosterone biosynthesis are shown in Tables 3 and 4, respectively. The method developed by Häusler *et al.* [5] was applied using ACTH-stimulated rat adrenal fragments in potassium containing buffer. As can be seen from the $IC_{50}$ values in Table 3, compound 1 inhibited corticosterone formation to a lesser extent than AG ($IC_{50}$: 140 and 50 μM). In this test compound (+)-1 is more active than its antipode. In the inhibition of aldosterone formation similar differences are observed (Table 4). In these experiments compound 1 also turned out to be a marginally weaker inhibitor than AG ($IC_{50}$: 130 and

Table 3. Inhibition of corticosterone formation by 1 and AG[a]

| Compound | Concentration (μM) | $n$ | % Of control[b] | $IC_{50}$ (μM) |
|---|---|---|---|---|
| 1 | 100 | 3 | 56 ± 7 | 140 |
| | 300 | 3 | 24 ± 2 | |
| (+)-1 | 100 | 4 | 45 ± 4 | <100 |
| | 300 | 4 | 28 ± 3 | |
| (−)-1 | 100 | 3 | 61 ± 9 | 170 |
| | 300 | 3 | 37 ± 4 | |
| AG | 30 | 9 | 68 ± 15 | 50 |
| | 100 | 4 | 28 ± 4 | |

[a]*In vitro*; rat adrenal slices; stimulation by ACTH. [b]Mean value ± SD.

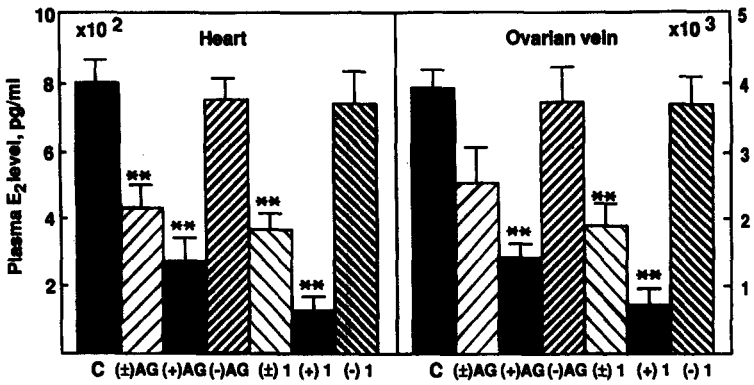


Fig. 3. Effect of compound 1 and AG on plasma $E_2$ level of the PMSG-pretreated SD rat ($n = 5$); blood taken from the ovarian vein as well as from the right ventricle 6 h after a single dose application of the compounds in a dosage of 0.31 mg/kg (1) and 1.25 mg/kg (AG). $^*P < 0.05$, $^{**}2P < 0.05$ (paired students $t$-test).

80 $\mu$M). No significant differences in the activities between the enantiomers of 1 are observed ($IC_{50}$: 125 and 135 $\mu$M).

*In vivo studies*

The *in vivo* activity of compound 1 and AG on the plasma $E_2$ level was determined using PMSG-stimulated female rats according to the procedure of Brodie *et al.* [24]. The $E_2$ concentration lowering effect 6 h after s.c. application of the inhibitor is shown in Fig. 3. There are only marginal differences in the inhibition values found in plasma from the right ventricle and the ovarian vein.

According to the enhanced aromatase inhibitory potency *in vitro*, compound 1 also shows a much stronger $E_2$ lowering effect *in vivo* than AG, which was tested at a 5-fold higher dosage. The (+)-enantiomers of both compound 1 and AG are more active than the corresponding racemic mixtures, while the levorotary enantiomers are almost inactive in the applied doses.

The antitumor activity of compound 1 and AG was determined using the DMBA-induced mammary carcinoma of the ovariectomized, testosterone-treated SD rat [14, 25]. This model mimicks the endocrine situation of a postmenopausal or ovariectomized woman. As can be seen from Table 5 and Fig. 4, the ovariectomy-induced regression of the tumors can be overcome by the administration of testosterone. A similar effect is obtained by androstenedione, whereas the nonaromatizable androgen dihydrotestosterone shows no effect. The testosterone (androstenedione) stimulation of tumor growth can be dose-dependently inhibited by aromatase inhibitors, and therefore is probably due to aromatization in peripheral tissues [26].

Table 4. Inhibition of aldosterone formation by 1 and AG[a]

| Compound | Concentration ($\mu$M) | $n$ | % Of control[b] | $IC_{50}$ ($\mu$M) |
|---|---|---|---|---|
| 1 | 100 | 3 | 51 ± 15 | 130 |
| | 300 | 4 | 28 ± 6 | |
| (+)-1 | 100 | 3 | 54 ± 6 | 125 |
| | 300 | 4 | 35 ± 4 | |
| (−)-1 | 100 | 4 | 56 ± 13 | 135 |
| | 300 | 4 | 32 ± 8 | |
| AG | 30 | 6 | 67 ± 6 | 80 |
| | 300 | 6 | 26 ± 5 | |

[a] *In vitro*; rat adrenal slices; stimulation by ACTH. [b] Mean value ± SD.

Table 5. Effect of compound 1 and AG on the DMBA-induced, hormone-dependent mammary tumor of the ovariectomized testosterone-treated SD rat (postmenopausal model)

| Treatment group[a] | Dose of inhibitor [mg/kg][b] | No. of tumors | | % Of tumors with | | | | % Change of tumor area[i,j] |
|---|---|---|---|---|---|---|---|---|
| | | B[c] | NT[d] | CR[e] | PR[f] | NC[g] | P[h] | |
| Control | | 24 | 1 | 83 | 17 | 0 | 0 | −98 |
| T | | 26 | 1 | 32 | 28 | 12 | 28 | −26[k] |
| AG + T | 20 | 24 | 0 | 86 | 5 | 5 | 4 | −81[k,l] |
| 1 + T | 12.3 | 32 | 0 | 81 | 19 | 0 | 0 | −96[l] |
| 1 + T | 6.2 | 25 | 0 | 66 | 25 | 9 | 0 | −84[k,l] |
| 1 + T | 2.5 | 28 | 0 | 83 | 4 | 6 | 7 | −79[k,l] |

[a] T: testosterone. [b] Dose of T: 20 mg/kg. [c] At the beginning of the experiment. [d] Occurring during the experiment. [e] CR: complete remission, tumor not palpable. [f] PR: partial remission, reduction of initial tumor size ⩾ 50%. [g] NC: no change, tumor size 51–150% of initial tumor size. [h] P: progression, tumor size > 150% of initial tumor size. [i] Average on the 28th day of treatment. [j] The U-test of Wilcoxon, Mann, and Whitney was used. [k] Significantly different from the control group ($\alpha = 0.01$). [l] Significantly different from the T group ($\alpha = 0.01$).

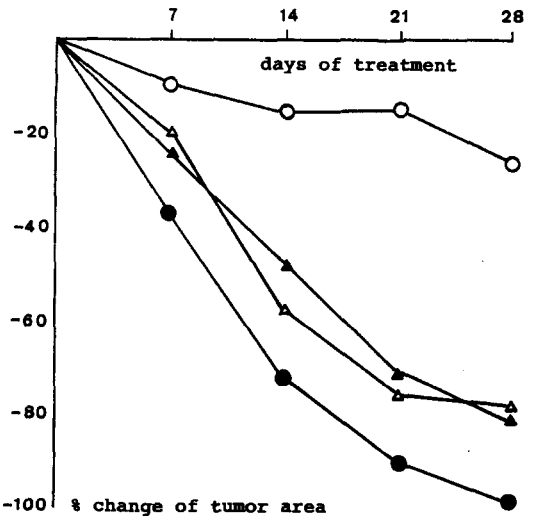


Fig. 4. Effect of compound 1 and AG on the tumor area of the DMBA-induced hormone-dependent mammary carcinoma of the ovariectomized, testosterone-treated SD rat (postmenopausal model): control (●——●), T (20 mg/kg) (○——○), T + AG (20 mg/kg) (▲——▲), T + compound 1 (2.5 mg/kg) (△——△). The compounds were applied 6 times per week.

The results in the DMBA experiment of AG and compound **1** confirm the findings of the PMSG experiment. Given at $\frac{1}{10}$ of the dose of AG, compound **1** is equieffective to AG in inhibiting the testosterone-stimulated tumor growth of the ovariectomized DMBA tumor bearing rat.

To obtain information about the possible CNS-depressive activity, compound **1** and AG were examined in the motility test [28] and the rotarod experiment [29] in mice. Even at very high doses (600 mg/kg) the cyclohexyl compound did not show any effect in these tests, whereas AG had an $ED_{50}$ of 62 mg/kg in the motility test and an $ED_{50}$ of 164 mg/kg in the rotarod experiment.

## DISCUSSION

The present study shows that compound **1** is a highly active aromatase inhibitor, whose inhibitory activity resides almost exclusively in its dextro isomer, compound (+)-**1**. The amino nitrogen of the new compound interacts with the central iron of cytochrome $P_{450}$ arom, while the rest of the molecule interacts with the substrate binding site at the apoprotein moiety of the enzyme. Thus, compound (+)-**1** should be a helpful scientific tool to elucidate the topography of the active site of aromatase, the crystal structure of which is still unknown.

*In vivo* the differences in the activities of AG and compound **1** are less dramatic. Nevertheless the *in vivo* properties of the new compound are remarkable. The racemate is approx. 10 times as strong as the parent compound AG in inhibiting mammary tumor growth in ovariectomized, testosterone-treated rats. In PMSG pretreated adult rats it is the dextro enantiomer, (+)-**1**, which is predominantly responsible for the $E_2$ lowering effect. Thus compound (+)-**1**, being more active as AG *in vivo* by a factor of about 20, should be more favorable for clinical use.

In this connection it is of great interest that compound (+)-**1** exhibits a specificity for aromatase as compared to other enzymes involved in steroid biosynthesis. Desmolase inhibition is reduced compared to AG and even more important the compound is less effective in lowering adrenal corticosterone and aldosterone production *in vitro*. With regard to the mineralocorticoid production, compound (+)-**1** is also superior to two other highly active nonsteroidal aromatase inhibitors recently being developed by others, CGS 16 949 A [32] and R 76 713 [33]. The two compounds are stronger inhibitors of aldosterone production compared to compound (+)-**1** [CGS 16 949 A: $IC_{50} = 0.6\,\mu M$; R 76 713: $IC_{50} = 12\,\mu M$; (+)-**1**: $IC_{50} = 125\,\mu M$]. They are about equieffective, however, in inhibiting corticosterone formation [CGS 16 949 A: $IC_{50} = 80\,\mu M$; R 76 713: $IC_{50} = 95\,\mu M$; (+)-**1**: $IC_{50} < 100\,\mu M$].

Based on the fact that compound (+)-**1** *in vitro* influences adrenal corticosterone and aldosterone production only in very high concentrations, it can be assumed that the compound does not affect adrenal steroid formation in those small concentrations sufficient to reduce estrogen levels. Thus a substitution of steroid hormones, as it is routinely applied in the therapy with AG, should not be necessary.

The fact that the therapy of hormone-dependent breast cancer in postmenopausal women with AG is second line, is predominantly due to its moderate tolerability, i.e. to its CNS depressive activity leading to severe side effects [6]. Therefore the finding that compound **1** does not show any CNS depressive activity is of great clinical relevance. In retrospect this finding is not really surprising since it has been known for a long time that barbituric acids with two cyclic substituents in the 5,5 position are not CNS active. The 3,3 substituted piperidine-2,6-diones, compound **1** and AG, are structurally related to the 5,5 substituted barbituric acids,

i.e. exchange of an ethylene (-$CH_2CH_2$-) moiety by a carboxylic acid amide (-CONH-) leads to the barbituric acids.

In conclusion the present data gives rise to the hope that compound (+)-1 might be a promising candidate for the treatment of estrogen-dependent diseases.

*Acknowledgements*—Thanks are due to the Verband der Chemischen Industrie, Fonds der Chemischen Industrie and to the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 234) who financially supported this work. We wish to thank Dr Nickel, Asta Pharma Frankfurt, for the performance of the motility test and the rotarod experiment, Lydia Gottswinter for technical assistance and Brunhilde Rischar for the preparation of the manuscript. Some of the experiments were performed in the labs of Professor Schönenberger at the Institute of Pharmacy, University of Regensburg. Thanks are due to Professor Schönenberger.

## REFERENCES

1. Siiteri P. K. and MacDonald P. C.: Role of extraglandular estrogen in human endocrinology. In *Handbook of Physiology, Section 7: Endocrinology, Vol. II. Female Reproductive System, Part 1* (Edited by R. O. Greep and E. B. Astwood). Williams and Wilkins, Baltimore (1973) pp. 615–629.
2. Brodie A. M. H. and Santen R. J.: Aromatase in breast cancer and the role of aminoglutethimide and other aromatase inhibitors. *CRC Crit. Rev. Oncol./Hemat.* **5** (1986) 361–396.
3. Bhatnagar A. S., Nadjafi C. and Steiner R.: Aromatase inhibitors in cancer treatment. In *Endocrine Management of Cancer* (Edited by B. A. Stoll). Karger, Basel, Vol. 2 (1988) pp. 30–42.
4. Häusler A., Schenkel L., Krähenbühl C., Monnet G. and Bhatnagar A. S.: An *in vitro* method to determine the selective inhibition of estrogen biosynthesis by aromatase inhibitors. *J. Steroid Biochem.* **33** (1989) 125–131.
5. Häusler A., Monnet G., Borer C. and Bhatnagar A. S.: Evidence that corticosterone is not an obligatory intermediate in aldosterone biosynthesis in the rat adrenal. *J. Steroid Biochem.* **34** (1989) 567–570.
6. Santen R. J., Samojlik E. and Worgul T. J.: Aminoglutethimide. Scientific profile. In *A Comprehensive Guide to the Therapeutic Use of Aminoglutethimide* (Edited by R. J. Santen and J. C. Henderson). Karger, Basel (1982) pp. 101–160.
7. Foster A. B., Jarman M., Leung C.-S., Rowlands M. G. and Taylor G. N.: Analogues of aminoglutethimide: selective inhibition of cholesterol side-chain cleavage. *J. Med. Chem.* **26** (1983) 50–54.
8. Foster A. B., Jarman M., Leung C.-S., Rowlands M. G., Taylor G. N., Plevey R. G. and Sampson P.: Analogues of aminoglutethimide: selective inhibition of aromatase. *J. Med. Chem.* **28** (1985) 200–204.
9. Daly M. J., Jones G. W., Nicholls P. J., Smith H. J., Rowlands M. G. and Bunnett M. A.: Synthesis and biochemical evaluation of analogues of aminoglutethimide based on phenylpyrrolidine-2,5-dione. *J. Med. Chem.* **29** (1986) 520–523.
10. Leung C.-S., Rowlands M. G., Jarman M., Foster A. B., Griggs L. J. and Wilman D. E. V.: Analogues of 3-ethyl-3-(4-pyridyl)piperidine-2,6-dione as selective inhibitors of aromatase: derivatives with variable 1-alkyl and 3-alkyl substituents. *J. Med. Chem.* **30** (1987) 1550–1554.
11. Batzl C. and Hartmann R. W.: Aromatase inhibitors. Syntheses and evaluation of potential mammary tumor inhibiting 4-alkyl-3-(4-aminophenyl)-3-ethyl-piperidine-2,6-diones. *Arch. Pharm. (Weinheim)* **320** (1987) 51–58.
12. Stanek J., Alder A., Bellus D., Bhatnagar A. S., Häusler A. and Schieweck K.: Synthesis and aromatase inhibitory activity of novel 1-(4-aminophenyl)-3-azabicyclo[3.1.0]hexane- and -[3.1.1.]heptane-2,4-diones. *J. Med. Chem.* **34** (1991) 1329–1334.
13. Hartmann R. W. and Batzl C.: Synthesis and evaluation of 4-alkylanilines as mammary tumor inhibiting aromatase inhibitors. *Eur. J. Med. Chem.* In press.
14. Hartmann R. W. and Batzl C.: Aromatase inhibitors. Synthesis and evaluation of mammary tumor inhibiting activity of 3-alkylated 3-(4-aminophenyl)piperidine-2,6-diones. *J. Med. Chem.* **29** (1986) 1363–1369.
15. Hartmann R. W., Batzl C., Pongratz T. M. and Mannschreck A.: Synthesis and aromatase inhibition of 3-cycloalkyl-substituted 3-(4-aminophenyl)piperidine-2,6-diones. *J. Med. Chem.* **35** (1992) 2210–2214.
16. Graves P. E. and Salhanick H. A.: Stereoselective inhibition of aromatase by enantiomers of aminoglutethimide. *Endocrinology* **105** (1979) 52–57.
17. Thompson E. A. and Siiteri P. K.: Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. *J. Biol. Chem.* **249** (1974) 5364–5372.
18. Hochberg R. B., van der Hoeven T. A., Welch M. and Lieberman S.: A simple and precise assay of the enzymatic conversion of cholesterol into pregnenolone. *Biochemistry* **13** (1974) 603–609.
19. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
20. Lineweaver H. and Burk D.: The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56** (1934) 658–666.
21. Bednarski P. J. and Nelson S. D.: Interactions of thiol-containing androgens with human placental aromatase. *J. Med. Chem.* **32** (1989) 203–213.
22. Zachariah P. K., Lee Q. P., Symms K. G. and Juchau M. R.: Further studies on the properties of human placental microsomal cytochrome *P*-450. *Biochem. Pharmac.* **25** (1976) 793–800.
23. Krebs H. A. and Henseleit K.: Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyler's Z. Physiol. Chemie* **210** (1932) 33–66.
24. Brodie A. M., Schwarzel W. C. and Brodie H. J.: Studies on the mechanism of estrogen biosynthesis in the rat ovary. *J. Steroid Biochem.* **7** (1976) 787–793.
25. Bayer H., Batzl C., Hartmann R. W. and Mannschreck A.: New aromatase inhibitors. Synthesis and biological activity of pyridyl-substituted tetralone derivatives. *J. Med. Chem.* **34** (1991) 2685–2691.
26. Hartmann R. W. and Batzl C.: Development of a postmenopausal rat mammary tumor model. *Pharm. Pharmac. Lett.* In press.
27. Kranzfelder G., Hartmann R. W., von Angerer E., Schönenberger H. and Bogden A. E.: 3,4-Bis(3'-hydroxyphenyl)hexane—a new mammary tumor-inhibiting compound. *J. Cancer Res. Clin. Oncol.* **103** (1982) 165–180.
28. Hölzl J. and Fink C.: Untersuchungen zur Wirkung der Valepotriate auf die Spontanmotilität von Mäusen. *Arzneim. Forsch. Drug Res.* **34** (1984) 44–47.
29. Gross F., Tripod J. and Meier R.: Zur pharmakologischen Charakterisierung des Schlafmittels Doriden. *Schweiz. Med. Wschr.* **13** (1955) 305–309.
30. Brodie A. M. H., Brodie H. J., Garrett W. M., Hendrickson J. R., Marsh D. A. and Tsai Morris C. H.: Effect of an aromatase inhibitor, 1,4,6-androstatriene-3,17-dione, on DMBA-induced mammary tumors in the

rat and its mechanism of action *in vivo*. *Biochem. Pharmac.* **31** (1982) 2017–2023.
31. Hodgson E. and Juchau M. R.: Ligand binding to human placental cytochrome *P*-450: Interaction of steroids and heme-binding ligands. *J. Steroid Biochem.* **8** (1977) 669–675.
32. Browne L. J., Gude C., Rodriguez H., Steele R. E. and Bhatnagar A.: Fadrozole hydrochloride: a potent, selective, non-steroidal inhibitor of aromatase for the treatment of estrogen-dependent disease. *J. Med. Chem.* **34** (1991) 725–736.
33. De Coster R., Wouters W., Bowden C. R., Van den Bossche H., Bruynseels J., Tuman R. W., Van Ginckel R., Snoeck E., Van Peer A. and Janssen P. A. J.: New non-steroidal aromatase inhibitors: focus on R 76 713. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 335–341.