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THE EFFECT OF THE AROMATASE INHIBITOR, ROGLETIMIDE (PYRIDOGLUTETHIMIDE), ON GUINEA PIG ADRENAL CELL STEROIDOGENESIS AND PLACENTAL MICROSOMAL AROMATASE ACTIVITY: COMPARISON WITH AMINOGLUTETHIMIDE AND CGS 16949A

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Summary—A dispersed guinea pig adrenal system has been used to study the effect of the aromatase inhibitor rogletimide (RGL) on adrenal steroidogenesis. The ACTH-stimulated release of cortisol, 17-hydroxyprogesterone (17-OHP) and androstenedione (A) was measured following exposure of adrenal cells to RGL, or the other aromatase inhibitors aminoglutethimide (AG) and CGS 16949A. RGL at concentrations sufficient to cause 80–90% inhibition of placental microsomal aromatase had no effect on the release of all three steroids. In contrast, AG at 10^{-5} M markedly reduced the output of all three steroids from these cells. CGS 16949A at 10^{-6} M reduced the output of cortisol and increased the concentration of 17-OHP and A. These results indicate that RGL is unlikely to cause the suppression of cortisol synthesis which has been noted to occur with AG and CGS 16949A during the treatment of breast cancer patients.

INTRODUCTION

The lowering of plasma oestrogen concentrations in postmenopausal women with breast cancer by way of selective aromatase inhibition is currently a highly active area of anticancer drug development. Since aromatase belongs to a class of cytochrome *P*-450 mixed function oxidase enzymes the problem is to obtain a drug of sufficient specificity. Aminoglutethimide (AG), an aromatase inhibitor now widely used to reduce circulating oestrogen concentrations in patients with advanced metastatic breast disease [1, 2], has the disadvantage that it suppresses adrenal glucocorticoid synthesis as a result of inhibiting 20,22 desmolase, 11β- and 21-hydroxylase [3]. This results in a compensatory increase in plasma levels of 17-hydroxyprogesterone (17-OHP) and androstenedione (A) [4, 5], an effect which is disadvantageous to the overall aim of oestrogen suppression. It therefore has to be administered with hydrocortisone as a glucocorticoid replacement therapy for maximum effectiveness [6] and therapeutic safety in the avoidance of adrenal insufficiency [7]. The recently developed compound CGS 16949A has been shown to be a far more potent inhibitor of aromatase than AG [8]. However, *in vitro* [9] and *in vivo* [10] studies have shown that this compound is also not totally specific towards the inhibition of aromatase.

In an attempt to overcome the lack of specificity of aromatase inhibition, Foster *et al.* [11] synthesized an analogue of AG, rogletimide [3-ethyl-3-(4-pyridyl)piperidine-2,6-dione] (RGL), previously known as pyridoglutethimide. Leung *et al.* [12] reported that this compound inhibited aromatase without reducing the activity of the cholesterol side-chain cleavage enzyme, 20,22 desmolase. The current study extends these early observations by using dispersed guinea pig adrenal cells to determine the effect of RGL on ACTH-stimulated steroidogenesis. Comparison is made with AG, and the more potent aromatase inhibitor, CGS 16949A. The effectiveness of each of these compounds as an inhibitor of placental microsomal aromatase is also determined.

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MATERIALS AND METHODS

Chemicals and reagents

[2,4,6,7-^{3}H]17-OHP was purchased from Amersham International. [1β-^{3}H]A (sp. act. 27.5 Ci/mmol) was obtained from New England Nuclear. The coenzyme, NADPH, for aromatase measurement was supplied by Sigma Chemical Co. Collagenase type I for adrenal cell dispersion was obtained from Worthington Biochemical. Bovine serum albumin (BSA) was supplied, protease free, by Miles Labs. RGL was kindly supplied by Dr M. Jarman (Department of Drug Development, CRC Labs, Sutton, Surrey). AG and CGS 16949A were supplied by Ciba–Geigy Pharmaceuticals. Minimal Essential Medium (MEM) containing Earl's salts, 20 mM Hepes buffer, but without L-glutamine (MEM-A) was provided by the Chester Beatty Institute of Cancer Research (Fulham Road, London). Synthetic ACTH was provided by Ciba-Geigy Pharmaceuticals and stored as a stock solution in 0.9% sodium chloride containing 5% BSA adjusted to pH 3.5 with 0.1 M HCl. All other chemicals and reagents were obtained from Sigma Chemical Co.

Adrenal cell preparations

The method used was an adaptation of that described by Lambert *et al.* [13]. A male guinea pig (Dunkin-Hartley, 500–650 g) was killed by cervical dislocation and the kidneys and attached adrenal glands were carefully removed. The kidney and fatty tissue was dissected away and the intact adrenal was cut in half, lengthwise. The two pieces of each adrenal were placed on a cooled Petri dish (4°C) and cut into cubes of about 1 mm^3 with a fine blade. The divided tissue was then placed into 10 ml MEM-A (4°C), previously gassed for 5–10 min with 95% O_2/5% CO_2. Fragments of tissue were washed ($\times 2$) with fresh media (MEM-A, 4°C) and were then poured into a small gas chamber (50 ml, with magnetic stirring bar). MEM (10 ml) containing 2 mg/ml collagenase (MEM-B) was added. The chamber was kept at 37°C and gassed with 95% O_2/5% CO_2. Cells were allowed to disperse for 10 min following which the medium was removed and discarded. A further 10 ml aliquot of MEM-B was added to fragments and dispersion was carried out for 10 min. Medium containing dispersed cells (supernatant 2) was removed and kept on ice. Cell dispersion was carried out for a further 2 cycles and supernatants 2–4 were finally centrifuged at 4°C for 5 min at 400 *g*. A portion of each supernatant was removed and discarded leaving 5 ml above the cell pellet and 1.5 ml MEM containing 2 mM ascorbate 0.5% BSA and 8 mM $CaCl_2$ (MEM-C, made up just prior to addition) was added. Starting with supernatant 3, cells were carefully resuspended (without air bubbles) and suspensions were filtered through 1 μm nylon mesh. The pooled cell suspension was centrifuged at 400 *g* for 5 min, the supernatant removed and 10 ml MEM-C was added to resuspend cells. Centrifugation was again performed for 5 min at 400 *g* and the pellet was resuspended in 3 ml MEM-C. Approximately equal aliquots of this suspension were then placed into two chambers of a multiwell plate (24 well) and left for 2 h in an incubation chamber flushed with 95% O_2/5% CO_2. Following this period, cells were resuspended by gentle flushing with covering medium, removed from the wells and centrifuged for 5 min at 400 *g*. The supernatant was removed and the pelleted cells were resuspended in 2 ml MEM containing 4 mM ascorbate, 0.5% BSA and 8 mM $CaCl_2$ (MEM-D). Cell number was determined with a haemocytometer using trypan blue to exclude counting non-viable cells. The number of cells in the preparation was adjusted to give between 2.5×10^6 and 5×10^6 cells/ml by the addition of MEM-D.

Adrenal cell incubations

To determine the basal production of steroids, portions of the cell suspension were added to wells of microtitre plates followed by 25 μl of a 10% solution of dimethylsulphoxide (DMSO) in MEM-A containing 0.5% BSA and 8 mM $CaCl_2$ (MEM-E) and 25 μl MEM-A alone. To observe the effect of ACTH on steroid output, 25 μl MEM-E containing 10% DMSO was added to 50 μl cell suspension followed by 25 μl ACTH (200 pg/ml in MEM-A). The effect of each of the three drugs on ACTH-stimulated steroidogenesis was determined in wells containing 50 μl cell suspension, 25 μl drug solution in MEM-E and 25 μl ACTH in MEM-A. Incubation was conducted for 90 min in an atmosphere of 95% O_2/5% CO_2.

Radioimmunoassay (RIA) of steroids

17-OHP and A were measured using direct RIAs (Biogenesis). Medium from each well was diluted 1:5 in steroid stripped human serum prior to assay. Cortisol was measured in the medium covering each of the cell layers by a

direct RIA using reagents provided by the Scottish Antibody Production Unit (SAPU; Candersham, Scotland). Standards for the measurement of cortisol were prepared in MEM-A and medium from each well was diluted 1:5 with MEM-A prior to assay.

Assay of aromatase activity in placental microsomes

The aromatase enzyme was measured by quantifying the amount of tritiated water released from radiolabelled A during aromatization to oestrone as described by Newton *et al.* [14]. Placental microsomes and NADPH (1 mM) were added in 0.1 M sodium phosphate buffer (pH 7.4) to assay tubes containing 0.5 μCi [1β-^{3}H]A and unlabelled aromatase inhibitors (10^{-10}–10^{-4} M final concentration), to give a final volume of 1 ml. After incubation for 1 h at 37°C, 0.5 ml of trichloroacetic acid was added followed by 1 ml of activated charcoal suspension (5% v/v). Following incubation for 30 min at 37°C, tubes and contents were centrifuged at 1500 *g* for 15 min at 4°C and radioactivity was determined in 1 ml aliquots of each supernatant. In order to determine IC_{50} values for each drug, counts observed at each concentration were calculated as a percentage of those observed in control tubes. Counts observed for assay blanks obtained for tubes where microsomes were replaced by phosphate buffer were <0.05% of the [1β-^{3}H]A added.

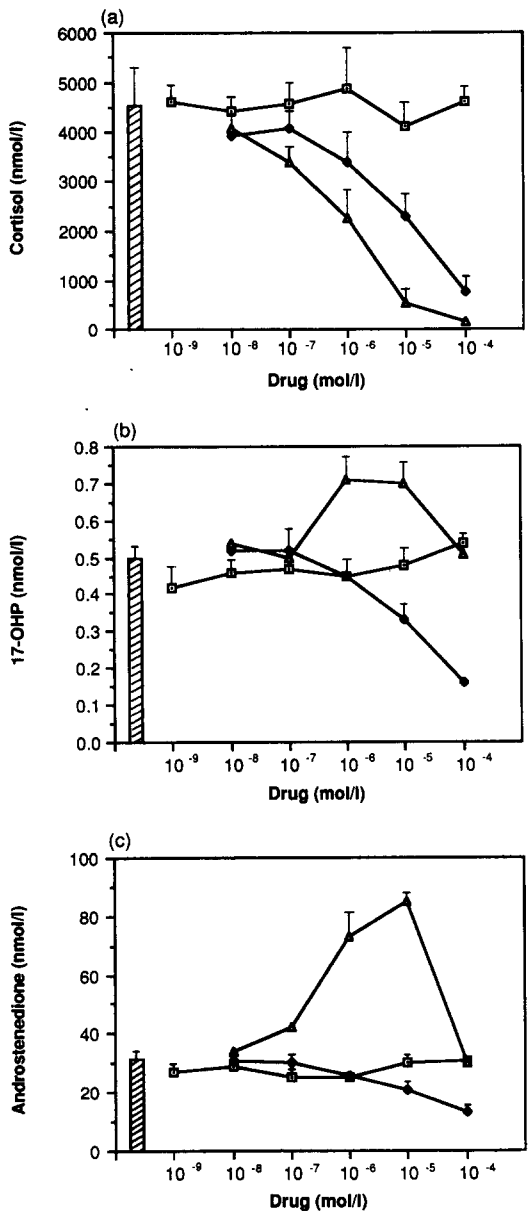

Fig. 1. The effect of RGL (▣), AG (◆) and CGS 16949A (△) on ACTH-stimulated cortisol (a), 17-OHP (b) and A (c) production by dispersed guinea pig adrenal cells. All values are expressed as steroid concentration in media covering cells and are mean ± SD for triplicates estimations. Hatched bars represent the steroid output in response to ACTH alone.

Table 1. Drug concentrations giving 50% inhibition of cortisol output by adrenal cells and aromatase activity in placental microsomes

Compound	Cortisol secretion IC_{50} (μM)	Aromatase activity IC_{50} (μM)
RGL	>100	19
AG	13	2
CGS 16949A	1	0.018

RESULTS

The effect of increasing concentrations of the three drugs on ACTH (50 ng/l) stimulated cortisol, 17-OHP and A secretion is shown in Figs 1(a–c), respectively. It is clear that RGL, over the entire concentration range tested, had no effect on the formation of any of the steroids measured. In contrast, AG and CGS 16949A at concentrations in excess of 10^{-6} M markedly reduced the output of cortisol by these cells. At 10^{-5} M this effect is statistically significant for both compounds ($P < 0.05$ and $P < 0.01$ for AG and CGS 16949A, respectively, test vs ACTH alone). Although 10^{-5} M AG also reduced concentrations of 17-OHP and A ($P < 0.05$ and $P < 0.01$ respectively), CGS 16949A at concentrations of 10^{-6} and 10^{-5} M markedly increased the concentration of these steroids in the media.

Table 1 shows IC_{50} values derived from Fig. 1(a) for the inhibition of cortisol output and also for aromatase activity measured in placental microsomes in response to the three drugs tested.

DISCUSSION

The results obtained from this study indicate that RGL has improved specificity over AG. However, it is important to consider the potency of aromatase inhibition when comparing dose-related inhibition of cortisol synthesis. At concentrations of RGL over the range 10^{-5}–10^{-4} M, shown in this study and others [11, 12] to inhibit aromatase activity in placental microsomes by 80–90%, no effect on ACTH-stimulated adrenal steroidogenesis could be detected. The results obtained here for the effect of RGL on adrenal steroid secretion are in good agreement with those obtained by Kitawaki *et al.* [15], where no effect on partially purified adrenal cytochrome *P*-450 steroid metabolizing enzymes could be demonstrated. The potency of RGL as an inhibitor of aromatase also appears to be comparable between the two studies since Kitawaki *et al.* [15] reported IC_{50} values in the range 10–19 μM. The current study gives an IC_{50} value for the inhibition of cortisol synthesis by AG of a similar order to that reported by Lambert *et al.* [13], who also used a dispersed guinea pig adrenal cell system. A more recent study by Lamberts *et al.* [16], where primary cultures of human adrenal cells were used, demonstrated that CGS 16949A significantly reduced the ACTH-stimulated output of cortisol at a concentration of drug comparable to that reported here (10^{-7} M). As demonstrated in the current study, these authors were also able to show that CGS 16949A caused a marked elevation in the output of 17-OHP and A, indicating a similar mechanism of action of this drug in both human and guinea pig adrenal cells. Although similar increases were not observed with AG in the current study, elevations in 17-OHP and A occur *in vivo*. This is due to compensatory increases in ACTH secretion from the pituitary sufficient to overcome the blockade at 20,22 desmolase. Unlike AG, CGS 16949A does not inhibit 20,22 desmolase. Therefore, in the present experimental system, inhibition of cortisol synthesis by CGS 16949A at the level of 11β-hydroxylase [16] allows the build up of precursors in the culture medium.

It is clear from the current study and several previous reports that *in vitro* RGL is a less potent aromatase inhibitor than AG [11, 12, 15]. Like AG, RGL appears to interact competitively with substrate for the binding site of the aromatase cytochrome *P*-450 [15]. There may, however, be a difference in the kinetic characteristics of enzyme, substrate and inhibitor interactions *in vitro* and *in vivo* as demonstrated for the enzyme, oestradiol dehydrogenase (E_2DH) from breast tumour cells [17]. Steroids shown to inhibit E_2DH isolated from breast tissue failed to inhibit the conversion of oestrone to oestradiol by cultures of growing malignant breast cells. A difference in potency in favour of AG over RGL measured using placental microsomes as a source of aromatase might not be apparent *in vivo*. Preliminary clinical data reveal that marked and near maximal suppression of plasma oestrogen concentrations occurs at an RGL dose level of 200 mg b.d. [18]. The degree of inhibition reported in this recent clinical study compares favourably with that reported by Harris *et al.* [4], for low dose AG treatment of advanced breast cancer patients. The discrepancy between these *in vivo* observations and the results presented in this paper, may reflect the insensitivity of plasma oestrogen measurement as a determinant of *in vivo* aromatase inhibition [19]. Further studies with a larger number of patients, together with the measurement of the degree of inhibition of peripheral aromatase *in vivo* [19] by different doses of RGL, are therefore required to select a maximally effective dose. These studies will also establish how well this drug is tolerated by patients with advanced breast cancer.

The current study suggests that RGL has the required endocrine specificity to allow further clinical testing. The original selection of the compound as not inhibiting 20,22 desmolase activity was on the basis that such inhibition would require glucocorticoid replacement as concurrent treatment. The current observations showing no significant effect on other enzymes involved in cortisol synthesis confirm this as an advantage over AG. The addition of this compound to the few effective antiendocrine agents for breast cancer therefore awaits the outcome of these clinical trials.

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