In Vivo and in Vitro Pharmacological Studies of Aminoglutethimide as an Aromatase Inhibitor


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Abstract

Use of steroid biosynthesis inhibitors to suppress estrogen production is a logical strategy in the treatment of women with hormone-dependent breast cancer. The clinical availability of aminoglutethimide as an inhibitor of cytochrome P-450-mediated steroid hydroxylations prompted study of the precise pharmacological and biochemical effects of this drug. Pharmacokinetic studies revealed that aminoglutethimide alters its own metabolic clearance rate as well as that of dexamethasone, a synthetic glucocorticoid. The metabolic clearance rates of other steroids such as hydrocortisone, medroxyprogesterone acetate, androstenedione, and estrone are not altered by aminoglutethimide. These findings led to the development of a practical regimen of escalating aminoglutethimide dosage in combination with hydrocortisone for treatment of patients with breast carcinoma. Further studies focused upon the biochemical mechanism of estrogen suppression with aminoglutethimide. In vivo, isotopic kinetic data demonstrated that aminoglutethimide inhibits peripheral aromatase by 95 to 98% in postmenopausal women. In vitro experiments indicated that aminoglutethimide can effectively block aromatase directly in human breast tumors as well. With respect to relative potency, aminoglutethimide is a 10-fold more potent aromatase inhibitor than is testololactone but is less potent than are 4-hydroxyandrostenedione and several brominated androstenedione derivatives. Taken together, these studies suggest that aminoglutethimide blocks estrogen production at three sites in women with breast carcinoma: the adrenal cortex, extraglandular peripheral tissues containing aromatase, and breast carcinoma tissue itself.

Introduction

While AG\textsuperscript{2} was initially introduced in the United States as an anticonvulsant, subsequent clinical observations demonstrated its potent inhibitory effects on adrenal steroid biosynthesis (3, 7, 9, 14). By binding to cytochrome P-450 complexes, AG blocks the cholesterol side-chain cleavage enzyme as well as the C-21, C-11, and C-18 steroid hydroxylases (4, 7, 9, 10, 23). Cash et al. (3) first conceived the use of AG as a form of “medical adrenalectomy” for treatment of metastatic breast carcinoma in patients who were otherwise candidates for surgical adrenalectomy. Griffiths et al. (8) later reported beneficial effects in 3 of 9 such women treated. Our group then studied the use of AG, focusing primarily on its precise pharmacological effects and mechanism of action. Since other antiepileptic agents such as phenobarbital were known to alter drug metabolism (2, 5), we first examined the effect of AG on the metabolic clearance of several synthetic and endogenous steroids as well as on its own metabolism. Stimulated by the studies of Thompson and Siiteri (21, 22), we then evaluated the ability of AG to block aromatase in vivo and in vitro and its relative potency compared to other aromatase inhibitors (18).

Our data demonstrated that AG accelerates its own metabolism as well as that of dexamethasone but does not alter the metabolic clearance of hydrocortisone, medroxyprogesterone acetate, androstenedione, and estrone. When given with replacement hydrocortisone, AG blocks estrogen synthesis at 3 sites: the adrenal cortex; extraglandular peripheral tissues containing aromatase; and breast carcinoma tissue itself.

MATERIALS AND METHODS

MCR

As described in detail previously, standard methods for determining MCR were used for the steroid hormones (18, 20). Briefly, \(^3\)H-steroids were injected i.v. as a single bolus or as constant infusions. Plasma samples were obtained at frequent intervals, and the \(^3\)H-steroids were quantitated after extensive purification and \(^{14}\)C recovery corrections. The MCR of AG was determined after a single p.o. administration by measuring plasma disappearance of unlabeled drug (12).

Aromatase Activity

In Vivo Studies. The method of MacDonald et al. (11) was used to measure the aromatization of androstenedione to estrone in vivo. Briefly, \(^1\)H-androstenedione (\(\Delta^4\)) and \(^{14}\)C-estrone (E\(_1\)) were infused under equilibrium conditions. The percentage of conversion (\(\rho\) value) of \(^1\)H-androstenedione to estrone was determined from the ratio of \(^{14}\)C estrone to \(^1\)H estrone in plasma after correction for recovery losses and exhaustive purification to make \(^{14}\)C: \(^3\)H ratios constant. The data were then confirmed in urine by measuring \(\rho\) values using 72-hr pools of urine.

In Vitro Studies. The \(^3\)H\(_2\)O method of Thompson and Siiteri (21) as modified by Reed and Ohno (15) and Weisz (24) was used to measure aromatization in placental microsomes, rat brain, and breast tumor homogenates. The exact technique involves incubation with \(^{1}\)H-\(\Delta^4\)androstenedione, separation of \(^3\)H\(_2\)O from nonaromatized steroid, and recovery and quantitation of \(^3\)H\(_2\)O. The validation of this assay for use in breast tumors will be published elsewhere.\textsuperscript{3} To compare inhibitory

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\textsuperscript{2}The abbreviations used are: AG, aminoglutethimide; MCR, metabolic clearance rate.

tors in microsomes, dose-response curves were constructed using identical incubation conditions for each compound. [1-3H]Androstenedione (8 pmol) was incubated with 10 µg of placental microsomes at room temperature for 20 min, resulting in an activity of about 50 pmol estrone formed per mg protein per hr. When studying inhibitors in breast tumor tissue, 400 to 1000 µg of crude homogenate were incubated with 80 pmol [1-3H]androstenedione for 60 min at 30°. Activities ranged from 10 to 60 pmol estrone formed per mg protein per hr. Consequently, 50% inhibitory concentrations of drugs can only be compared in experiments where conditions are identical.

Steroid Radioimmunoassays

Steroids were measured after plasma extraction and Celite column chromatography by standard radioimmunoassay methods as described previously. Assay sensitivity, precision, recoveries, and normal ranges for the various steroids have been documented in detail from our laboratory (17).

Aromatase Inhibitors

The AG used was a gift from the CIBA-GEIGY Corp., Ardsley, N. Y. Dr. Yoshio Osawa (Medical Foundation of Buffalo, Inc., Buffalo, N. Y.) provided the brominated and nitroso compounds. Testololactone was provided by Dr. Yoshio Osawa (Medical Foundation of Buffalo, Inc., Buffalo, N. Y.). Dr. Angela Brodie (University of Maryland School of Medicine, Baltimore, Md.) provided the 4-hydroxyandrostenedione.

Results

Effects of AG on MCRs

AG Metabolism. AG accelerated its own metabolism from a basal value of 2.6 ± 0.3 (S.E.) liters/24 hr to 5.3 ± 1.4 liters/24 hr after 1 to 2 weeks of drug administration (Table 1). This effect may partially explain the resolution of soporific side effects of AG which are most severe during the initial administration of drug. Based upon this finding, we currently administer 500 mg of AG daily for 2 weeks and then escalate the dose to 1000 mg daily thereafter.

Synthetic and Endogenous Steroids. AG markedly accelerates the metabolism of the synthetic glucocorticoid, dexamethasone, from basal values of 145 ± 26.6 liters/24 hr to 568 ± 127 liters/24 hr (p < 0.02) after 2 weeks of drug administration (Table 1). In contrast, the MCR of the endogenous steroid hydrocortisone is not altered or is altered only minimally [basally 159 ± 22 liters/24 hr; treatment, 192 ± 37 (not significant)]. Consequently, hydrocortisone is preferred to dexamethasone as replacement glucocorticoid in patients receiving AG. The MCR of medroxyprogesterone acetate, androstenedione, and estrone did not differ before or during treatment with AG (Table 1).

Effects of AG on Aromatase

In Vivo. The p value of Δ4 to E1 conversion, a direct in vivo measurement of aromatase, was determined prior to and during AG treatment in women with metastatic breast carcinoma. Blood studies showed that AG inhibited this enzyme by 98% from 1.65 ± 0.28% to 0.04 ± 0.01% (p < 0.01; Chart 1). As further confirmation of this observation, we found the p value of (Δ4-E1) in 2 of these same women using the urinary method to be inhibited by 95% (Table 2). Demonstration of radiochemical purity (by measuring constant 3H:14C ratios) served to validate the steroid isolation techniques used. Thus, at the blood levels achieved (i.e., 30 µM), AG blocks extraglandular aromatization nearly completely (i.e., by 95 to 98%).

Another means of estimating the degree of aromatase inhibition was to measure plasma levels of estrone and estradiol in women receiving AG. The control group for this analysis was a group of postmenopausal women who had undergone surgical adrenalectomy to remove endogenous estrogen sources. As shown in Chart 2, estrone and estradiol fell to similar levels in response to either AG-hydrocortisone or surgical adrenalectomy. These data provide further evidence of aromatase suppression with AG.

In Vitro. Initial studies identified the frequency with which aromatase is present in individual breast tumor homogenates. Of 25 tumors studied (Chart 3), 21 contained detectable amounts of aromatase. The range of activity varied from a low of 5 pmol estrone per g protein per hr to a high of 80 pmol estrone per g protein per hr (Chart 3). It should be noted that,

![Chart 1: Effect of AG on aromatization of androstenedione (Δ4) to estrone (E1) as measured in blood. Points, individual patients studied before (basal) and during treatment with 1000 mg of AG and 40 mg of hydrocortisone daily. Horizontal lines, mean value for all patients. Downward sloping lines connect individual studies before and during treatment. From Santen et al. (18), reproduced with permission of the Journal of Clinical Endocrinology and Metabolism.](image)

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of AG on drug and steroid MCRs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
<td><strong>Before AG (liters/24 hr)</strong></td>
</tr>
<tr>
<td>AG</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>145 ± 26.6</td>
</tr>
<tr>
<td>Medroxyprogesterone acetate</td>
<td>2096 ± 314</td>
</tr>
<tr>
<td>Cortisol</td>
<td>159 ± 22</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>1572 ± 241</td>
</tr>
<tr>
<td>Estrone</td>
<td>1897 ± 221</td>
</tr>
</tbody>
</table>

* AG administered for at least 1 week.

a Mean ± S.E.

b NS, not significant.
AG as an Aromatase Inhibitor

Table 2

Effect of AG on aromatization: urine method

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Pretreatment</th>
<th>During treatment (1000 mg AG daily)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>^14C (cpm)</td>
<td>^3H (cpm)</td>
</tr>
<tr>
<td>Subject 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Celite</td>
<td>50</td>
<td>237</td>
</tr>
<tr>
<td>TLC I</td>
<td>168</td>
<td>191</td>
</tr>
<tr>
<td>TLC II</td>
<td>340</td>
<td>394</td>
</tr>
<tr>
<td>TLC III</td>
<td>298</td>
<td>336</td>
</tr>
<tr>
<td>TLC IV, Estrone acetate</td>
<td>298</td>
<td>292</td>
</tr>
<tr>
<td>p (%)</td>
<td></td>
<td>1.91</td>
</tr>
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<td></td>
<td>38</td>
<td>626</td>
</tr>
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<td>113</td>
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<tr>
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<td>290</td>
<td>39</td>
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<tr>
<td>Subject 5</td>
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<td></td>
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<tr>
<td>Celite</td>
<td>232</td>
<td>574</td>
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<tr>
<td>TLC I</td>
<td>809</td>
<td>615</td>
</tr>
<tr>
<td>TLC II</td>
<td>771</td>
<td>580</td>
</tr>
<tr>
<td>TLC III</td>
<td>674</td>
<td>503</td>
</tr>
<tr>
<td>TLC IV, Estrone acetate</td>
<td>1343</td>
<td>1000</td>
</tr>
<tr>
<td>p (%)</td>
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<td>1.08</td>
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<td></td>
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<td>53</td>
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<td></td>
<td>1024</td>
<td>89</td>
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</tbody>
</table>

a Counts marked for spillover but not for aliquot volume.
b The ^14C: ^3H spillover ratio was 0.088 for this experiment.

at minimally detectable levels of aromatase activity, ^3H2O counts from the tumors were at least 2-fold higher (120 to 140 cpm) than were background counts of buffer blanks (60 to 80 cpm) or blanks using maximum concentrations of aromatase inhibitor (60 cpm).

In 6 breast tumor homogenates, AG inhibited aromatase in a dose-dependent fashion with 50% inhibition at a concentration of 13 μM (Chart 4). The aromatase in human breast tumors appeared similar to that from human placenta and rat hypothalamic homogenates in its inhibitory response to AG. Dose-response curves (experimental conditions were identical for each tissue except for the fact that placental preparations contained 125 ng of microsomal protein versus 400 to 1000 μg of crude homogenate for the breast tumors) revealed that...
AG produced 50% inhibition of placental aromatase (6 µM) and of hypothalamic aromatase (6 µM) (Chart 4). In addition, the dose-response slopes for human placenta, breast carcinoma, and rat brain aromatase were similar.

Relative Potency of AG as an Aromatase Inhibitor

Two aromatase inhibitors are currently available for clinical use in the United States, AG and testololactone. We examined the relative potency of AG versus testololactone and also versus compounds currently in the preclinical phase of testing. Because of the limited availability of breast tumors, placental microsomes served as the source of aromatase for these comparisons. All compounds were tested under conditions of identical enzyme and substrate concentrations. In this system, AG inhibited aromatase at 10-fold lower drug concentrations than did testololactone, on the other hand, was 30-fold more potent than was AG and 300-fold more than was testololactone. Combinations of AG with testololactone or 4-hydroxyandrostenedione produced additive but not synergistic effects (data not shown). Brominated compounds such as 6a-bromoandrostenedione, 6β-bromoandrostenediol, and 16-bromoandrostenetriol were 10- to 100-fold more potent than was AG. A series of nitrogen-containing drugs such as 17-imino-3-methoxy-3,5-androstanediene were much less potent (data not shown).

Discussion

Previous data from several investigative groups demonstrated that AG inhibits adrenocortical secretion of the ketosteroids, glucocorticoids, and mineralocorticoids but incompletely blocks androgen production (7, 9, 13, 14, 17) (Chart 6). Close recent observations indicate that AG also acts at 2 sites distal to the adrenal, breast cancer tissue aromatase (Site II) and the aromatase from normal extraadrenal tissues such as fat, muscle, and liver (1, 18, 21) (Chart 7, Site III). The concentrations of AG achieved in plasma (i.e., 30 µM) appear sufficient to block aromatase effectively at Sites II and III (12). Direct isotopic kinetic studies indicate that Site 3 is inhibited 95 to 98% by AG given in standard doses (i.e., 1000 mg daily). Indirect in vitro studies (Chart 4) predict a similar degree of inhibition in the tumor itself (Site II). Thus, it is possible that the major effect of AG is a direct blockade of estrogen synthesis by the breast carcinoma itself. Comparison of tissue levels of estrone and estradiol measured by gas liquid chromatography-

**Chart 5.** Comparison of aromatization inhibition produced by 4-hydroxyandrostenedione, AG, and testololactone. Shaded area, 95% confidence limits of inhibition in homogenates incubated without inhibitor. Note that substrate concentrations and aromatase activities differ in this experiment and in Chart 4. Consequently, absolute inhibitory concentrations cannot be compared between Charts 4 and 5.

**Chart 6.** Diagrammatic representation of the effect of AG on conversion of cholesterol to pregnenolone (cholesterol side-chain cleavage enzyme), on 11β-hydroxylation (conversion of 11-deoxycorticosterone to corticosterone), and on 18-hydroxylation (corticosterone to aldosterone production). AG also facilitates the conversion of Δ5- to Δ4-steroids. The overall effect is the inhibition of ketosteroids, glucocorticoids, and mineralocorticoids with incomplete suppression of androgen production. Preg, pregnenolone; Prog, progesterone; DOC, 11-deoxycorticosterone; 17-OH Preg, 17-hydroxyprogrenolone; 17-OH Prog, 17-hydroxyprogesterone; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate.

**Chart 7.** Diagramatic representation of the 3 sites of AG action. Site I, inhibition of adrenal steroidogenesis. Interruption of cortisol production necessitates replacement with exogenous hydrocortisone. Site II, blockade of aromatization directly in breast carcinoma tissue. Site III, inhibition of aromatization in extraglandular tissues which include liver, fat, and muscle predominantly.
mass spectrometry with plasma concentrations during AG therapy are necessary to study this question directly.

Measurable levels of plasma estrogen persist after AG treatment in postmenopausal women. Does this observation suggest that the aromatase inhibition produced by AG is not complete in such patients? Contrary evidence from isopic kinetic studies supports the concept of nearly total (i.e., 95 to 98%) blockade of aromatization. Furthermore, estrogen levels in postmenopausal women after surgical adrenalectomy or in women after both oophorectomy and adrenalectomy are similar to those found in women receiving AG (25). These additional data also suggest that AG completely blocks endogenous estrogen production. It is possible that the residual estrogens found after surgical ablative therapy or AG reflect dietary estrogens or precursor hormones. Further studies using synthetic diets and also more potent aromatase inhibitors such as 4-hydroxyandrostenedione are required to identify the mechanisms for residual estrogen production.

Our pharmacological studies demonstrated that AG exerts a wide range of metabolic effects in patients. These include alteration of its own and of dexamethasone metabolism (Table 1), blockade of thyroxine synthesis (19), and the facilitation of the 3β-ol-dehydrogenase, Δ4-Δ5-isomerase pathway (18), among others. Studies by other investigators showed that the genetically controlled acetylator function, which controls rate of 4-hydroxylation of androstenedione, is required to identify the mechanisms for residual estrogen production.

Our discussion agrees with that of other workers.

Acknowledgments

The authors wish to thank Matilda Stover for her assistance in the studies of MCRs. Marlene Thompson provided excellent secretarial assistance in the preparation of this manuscript.

References


Discussion

Dr. Segalloff: I would like to make one point about Dr. Santen’s last comment. Since we are talking about eventually treating breast cancer, if we’re getting an additive effect which is a great deal more inhibition of aromatization, and this is really what is effective in breast cancer, you have made a “10-strike.” It doesn’t have to be synergistic.

Dr. Santen: This hinges on how well we can determine the degree of inhibition of estrogen production. My own feeling is that aminoglutethimide and probably testolactone both inhibit aromatization effectively as measured by isopic kinetic studies. Our problem is the ability to measure very low levels of estrogen in patients that are being treated. I think we need to develop better methods of assessing the level of production of estrogen during administration of these compounds so that we can adequately study the additive situation. We have demonstrated so many drug-drug interactions between aminoglutethimide and glucocorticoids that it boggles the mind to think of the possibilities of drug-drug interactions between Tecslac and aminoglutethimide. But it remains an interesting question.

Dr. Nattolfin: It perhaps adds a different dimension if I talk to you about ovarian cancer. Ovarian cancers are the most frequent cause of death from genital cancer in women, and for some years Drs. Schwartz and Eisenfeld in our department have been looking at receptor binding. Essentially, half of all ovarian cancers have estrogen and now progesterin receptors, often in a level that is found in breast cancers. They started a series of patients on tamoxifen, and the drug extended disease-free intervals but has not produced cures. Recently, a medical student in our laboratory, Ron Voidess, began to look at aromatization and to
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Cancer Res 1982;42:3353s-3357s.

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