Response of Nitrosomethylurea-induced Rat Mammary Tumor to Endocrine Therapy and Comparison with Clinical Response

Jessica R. Wilkinson, Judith C. Williams, David Singh, Paul E. Goss, Douglas Easton, and R. Charles Coombes

ABSTRACT

We have compared the response of the N-methyl-N-nitrosourea-induced rat mammary tumor to various endocrine agents with response in patients with breast cancer. To do this, we have induced tumors in 228 animals (65% of intact rats developed tumors but only 14% of ovariec-tomized rats developed tumors). In intact rats, 4-hydroxyandrostenedione, tamoxifen, and a combination of tamoxifen, aminoglutethimide (AG), and danazol induced significant tumor regression ($P < 0.001$, $P = 0.01$, $P = 0.04$, respectively), whereas danazol, AG, and trilostane were ineffective when used singly. Further investigation showed that the inactivity of AG in the rat was due to extensive acetylation of this compound.

In order to mimic postmenopausal breast cancer, we ovariectomized rats after N-methyl-N-nitrosourea administration. In ovariec-tomized animals, AG was again ineffective in inducing tumor regression but 4-hydroxyandrostenedione was highly active when compared to controls ($P = 0.002$).

Comparison with response of this model to endocrine therapy with response in patients indicates that this has good predictive capacity since it shows that agents which have minor activity in human breast cancer such as danazol and trilostane are inactive in the model.

The intact rat model does not, however, predict whether a drug will be useful for pre- or postmenopausal patients. We recommend that before committing large numbers of patients to clinical trials on the basis of this model, the metabolism of new compounds should be compared in humans and rats.

INTRODUCTION

Endocrine therapy has become more important in recent years in the management of breast cancer because of ease of administration and reversibility of compounds such as tamoxifen and AG (1, 2). In addition, tamoxifen has been reported to have a beneficial effect in delaying relapse after surgery for primary breast cancer (3). Furthermore, selection of patients is possible by detecting ER in breast carcinomas since this predicts which patients are likely to respond to endocrine therapy (4). Most patients, however, relapse after responding to treatment and this reinforces the need for a reappraisal of our strategy in developing and selecting agents for clinical use and in designing rational combinations since it has been shown that multiple agent therapy can increase response rates in patients with advanced breast cancer (5).

To date, agents for clinical evaluation have been selected on the basis of their activity in rats bearing rat mammary tumors induced by either DMBA or NMU. NMU-induced tumors are biologically similar to hormone-responsive human breast carcinomas in that they contain significant amounts of ER and regress on ovariectomy (6). In the establishment of animal models of breast cancer to do both fundamental studies and test for more satisfactory treatment regimens, the ability of animal tumor cells to metastasize is desirable. McCormick et al. (7) reported that a single dose of NMU induced mammary cancers and metastasis to spleen, liver, and lung. We (8) have found upon histological examination of rats treated with three NMU injections no evidence of metastases of either mammary or other primary tumors. No prospective study has been carried out to compare response in rats and patients to a variety of endocrine agents. We have used the NMU-induced rodent tumor model to carry out such a comparison and to determine whether it would be possible to rank various therapies in order of effectiveness. We have also carried out studies of drug metabolism where a correlation does not exist.

MATERIALS AND METHODS

Antitumor Studies

Animals

Premenopausal Model. Inbred virgin female Ludwig/Wistar/Olac rats (OLAC 1976, Ltd., Oxon, England) were kept at 19°C in isolators with a regimen of 12 h light/day. They were fed CRM diet (Labsaw, Croydon, England) and received water ad libitum. NMU (Sigma Chemical Co., London, England) was dissolved in distilled water at 12.5 mg/ml and adjusted to pH 5.4 with acetic acid. Three hundred 50-day-old rats, in batches of 50 animals, were given 3 injections of 0.5 ml NMU/rat (5 mg/100 g body weight) via the tail vein on days 0, 14, and 28. The animals were transferred to the Institute, where they were kept at 22–23°C with a minimum of 8 h light/day and fed SDS diet (Special Diet Services, Ltd., Witham, England).

Postmenopausal Model. Two hundred fifteen virgin female Ludwig/Wistar/Olac rats were housed and given injections under the same conditions as described above. Bilateral ovariectomy was carried out between 14 and 21 days after the last NMU injection.

Treatment of Tumor-bearing Animals

All rats were treated with daily injections of endocrine agents 5 days/week for 4 weeks and then left untreated for a further month (Table 1). Control animals were given injections of vehicle only and housed under identical conditions. Tumor growth was assessed by measuring tumor diameter in 3 dimensions. The tumors were considered growing (or stable) when the diameter was increasing. The different doses of each compound were selected to give a range of effects that could be readily assessed. The responses that were scored and analyzed were the degree of tumor regression and the time to tumor reappearance. The two parameters were scored independently and the mean values were calculated.

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1 Recipient of a Clinical Fellowship from the Cancer Research Campaign.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: AG, aminoglutethimide; DMBA, dimethylbenzanthracene; NMU, nitrosomethylurea; ER, estrogen receptor; 4-OHA, 4-hydroxyandrostenedione; HGX, hydroxyaminoglutethimide.
treated in groups of 5–10 animals over a 2-year period.

**Tumor Volume Analysis**

At each time t, the volume of a given tumor was estimated from two measured diameters $d_1$, $d_2$, by

$$ V = \frac{1}{6} \pi \left( \frac{d_1 + d_2}{2} \right)^3 $$

and these volumes were summed over all tumors to give an estimate $V$, of the total volume of tumor for a given animal at time t. Given that some of the animals died before the first time at which they were to be assessed (21 days), it was difficult to analyze the changes in tumor volume parametrically, and instead animals were categorized into three groups: (a) those with 50% or greater reduction in total tumor volume; (b) those with 0–50% reduction in tumor volume; and (c) those with an increase in tumor volume. All animals who died before 21 days were included in this last group. Analysis has thus been based on the number of animals in each of these three groups and treatments were compared using the $\chi^2$ test for trend.

**Metabolism of Aminogluthethimide**

Animals selected were non-tumor-bearing female virgin Ludwig/Wistar/Olac rats. Metabolism cages were used for 24-h urine collections. Three animals received daily s.c. injections of standard steroid vehicle alone and a further three animals received daily injections of AG (50 mg/kg in standard steroid vehicle) for 2 weeks. Prior to and after 2 weeks of chronic AG therapy, 24-h urines were collected. Urine was analyzed by a method published previously (9) for the presence of the induced metabolite HXAG and compared to its presence in the urine of three patients similarly treated with 1 g AG p.o. daily for 3 to 6 weeks.

**RESULTS**

**Tumor Incidence.** Sixty-five % (193 of 300) of intact rats given NMU developed palpable tumors at 4–5 months of age. Tumors were found to be discrete, and 1–6 (mean, 2) tumors were found in each rat. Unfortunately only 35 of 215 (16%) of ovariectomized animals developed tumors when rats were 6–7 months old. In the postmenopausal rats, tumors were not discrete and rarely more than one tumor per rat was observed. All tumors were examined histologically and this confirmed that they were mammary tumors, similar to those found in intact rats. All tumors were found to contain significant (>15 fmol/mg cytosol protein) quantities of ER.

**Results of Therapy in Premenopausal Rats.** Table 2 shows the results of therapy in 193 intact animals. All groups have been compared with the control animals with respect to the reduction of tumor volume.

Ovariectomy ($P < 0.001$), tamoxifen ($P = 0.01$), the combination of tamoxifen, AG, and danazol ($P = 0.04$), and 4-OHA, both alone ($P < 0.001$) and when combined with tamoxifen ($P = 0.001$), cause tumor regression (Table 2, Groups 2, 3, 6, 8, and 11). AG, danazol, and trilostane do not cause tumor regression (Table 2, Groups 4, 5, and 7).

Estradiol alone does not cause tumor regression ($P = 0.53$). Using logistic regression to investigate the effect of estradiol or perphenazine on 4-OHA and the effect of estradiol or perphenazine on ovariectomy on tumor regression, we found that the concomitant administration of estradiol or perphenazine or 4-OHA or ovariectomy reduces the effect of tumor regression of 4-OHA or ovariectomy (Table 2, Groups 9, 10, 12, and 13; all $P$ values $< 0.001$).

**Results of Therapy in Postmenopausal Rats.** Table 3 shows the results of therapy in 35 animals. As in Table 2, both groups have been compared with the control animals. The response to AG is poor in both pre- and postmenopausal rats ($P = 0.87$ and 0.19, respectively) whereas 4-OHA causes significant tumor regression in all animals tested in both models ($P < 0.001$ and 0.002, respectively).

**Metabolism of Aminogluthethimide in Rats and Patients.** Metabolism of AG in rats and humans was found to be different. Thus, HXAG, an inactive metabolite, was found to constitute 25% of AG in the 24-h urine of patients; approximately 40% of AG was present as the parent compound. In contrast, the urine of treated rats showed no evidence of HXAG, and other studies (10) have shown that the compound undergoes virtually complete acetylation. Such a metabolite in inactive *in vitro*.

**Comparison with Results of Therapy in Patients.** Response rates to various endocrine therapies administered to patients are shown in Table 4 and are compared with the findings in the rodent NMU-induced tumor model. The data derived from patients have mostly been obtained from our own unit, with the exception of the results of ovariectomy in pre- and postmenopausal patients (11) and of tamoxifen and AG in premenopausal patients (2, 12).

It can be seen that the correlation is moderately good for premenopausal women. The exception to this is 4-OHA which is thought to be inactive in premenopausal women yet is very active in rats. In the postmenopausal model, AG, which is extremely active in patients (13), is found to be inactive. 4-OHA activity, however, correlates well with its efficacy in postmenopausal patients (14).
### MODEL OF ENDOCRINE THERAPY

#### Table 2 Results of endocrine therapy in premenopausal rats

All rats were treated for 4 weeks, and tumor size was recorded weekly by measuring the two largest diameters using Vernier calipers. From these diameters the total tumor volume was estimated for a given animal. The changes in total tumor volume were categorized into three groups: (a) those with 50% or greater reductions; (b) those with 0-50% reductions; and (c) those with an increase.

<table>
<thead>
<tr>
<th>No. of rats whose total tumor volume shows</th>
<th>Regression</th>
<th>Progression</th>
<th>P (comparison with controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50-100%</td>
<td>&lt;50%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1  Control</td>
<td>26</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>2  Ovariectomy</td>
<td>12</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td>3  Tamoxifen</td>
<td>18</td>
<td>38</td>
<td>1</td>
</tr>
<tr>
<td>4  Aminoglutethimide</td>
<td>11</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>5  Danazol</td>
<td>17</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td>6  Tamoxifen-aminoglutethimide-danazol</td>
<td>18</td>
<td>38</td>
<td>2</td>
</tr>
<tr>
<td>7  Trilostane</td>
<td>10</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>8  4-OHA</td>
<td>10</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>9  4-OHA + perphenazine</td>
<td>11</td>
<td>29</td>
<td>1</td>
</tr>
<tr>
<td>10 4-OHA + estradiol</td>
<td>12</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>11 4-OHA + tamoxifen</td>
<td>10</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>12 Ovariectomy + perphenazine</td>
<td>10</td>
<td>29</td>
<td>1</td>
</tr>
<tr>
<td>13 Ovariectomy + estradiol</td>
<td>12</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>14 Estradiol</td>
<td>11</td>
<td>37</td>
<td>0</td>
</tr>
</tbody>
</table>

Total 193 421

#### Table 3 Results of therapy in postmenopausal rats

Rats were given s.c. injections of 4-OHA or AG for 4 weeks. Procedures were the same as for Table 2.

<table>
<thead>
<tr>
<th>No. of rats whose total tumor volume shows</th>
<th>Regression</th>
<th>Progression</th>
<th>P (comparison with controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50-100%</td>
<td>&lt;50%</td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>1  Controls</td>
<td>6</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>4  Aminoglutethimide</td>
<td>17</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>8  4-OHA</td>
<td>12</td>
<td>17</td>
<td>10</td>
</tr>
</tbody>
</table>

Total 35 43

#### Table 4 Comparison of rat model response rates with breast cancer patients

These results show that both Ovariectomy and tamoxifen are effective in premenopausal patients and also in the animal model. 4-OHA is effective in the postmenopausal model but aminoglutethimide is not, due to extensive acetylation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Regression rates (%)</th>
<th>Significant difference from controls</th>
<th>No. of patients</th>
<th>Response rate (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premenopausal patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>12</td>
<td>100</td>
<td>Yes</td>
<td>36</td>
<td>33</td>
<td>21</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>18</td>
<td>72</td>
<td>Yes</td>
<td>44</td>
<td>27</td>
<td>22</td>
</tr>
<tr>
<td>Aminoglutethimide</td>
<td>11</td>
<td>18</td>
<td>No</td>
<td>12</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Danazol</td>
<td>17</td>
<td>18</td>
<td>No</td>
<td>14</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Trilostane</td>
<td>10</td>
<td>0</td>
<td>No</td>
<td>5</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Postmenopausal patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminoglutethimide</td>
<td>17</td>
<td>24</td>
<td>No</td>
<td>213</td>
<td>28</td>
<td>11</td>
</tr>
<tr>
<td>4-OHA</td>
<td>12</td>
<td>100</td>
<td>Yes</td>
<td>36</td>
<td>39</td>
<td>12</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Currently DMBA- or NMU-induced rat mammary tumors in intact rats constitute the standard systems for selecting possible endocrine agents for use in patients with breast cancer. The responses to ovariectomy and 4-OHA appear qualitatively different from those induced by tamoxifen and tamoxifen-aminoglutethimide-danazol, but all show significant regression compared with controls. From these studies on animals, we can rank various therapies in order of effectiveness. 4-OHA and ovariectomy showed marked tumor regression, tamoxifen and tamoxifen-aminoglutethimide-danazol induced significant tumor regression but showed lower regression rates compared with 4-OHA and ovariectomy, whereas danazol, trilostane, and aminoglutethimide proved ineffective in this animal model. In premenopausal breast cancer patients only ovariectomy and tamoxifen are effective and similar results are seen in rats; on the other hand, 4-OHA is ineffective in premenopausal patients but causes tumor regression in this model; this is thought to be a dose-related phenomenon in patients. Considering the intact tumor-bearing rat as a model for therapy of postmenopausal patients, neither trilostane nor danazol-treated animals differ from controls, and it is known that danazol has only minor activity and trilostane has no activity in postmenopausal patients (15, 16). Moreover, tamoxifen and 4-OHA are effective in rats and correlate well with clinical responsiveness in patients. Thus, there is a good correlation with results in postmenopausal patients.

There are some possible deficiencies in the “postmenopausal” model. These include the low frequency of tumors in ovariectomized animals restricting our ability to test a wide variety of endocrine therapies and the contrast between the sudden ces-
sation of ovarian function resulting from organ ablation and the natural menopause in women which is a gradual drawn out process. In order to develop a more appropriate postmenopausal rat model we are at present performing studies using older rats and genetically obese animals and varying the timing of surgery. However, there were sufficient animals to allow us to evaluate 4-OHA and AG; 4-OHA is active but AG has no effect. The results from studies of drug metabolism may help to explain the discrepancy in response rates between rats and humans. In rats N-acetylation is a major inactivation route of AG (10) accounting for >90% of urinary metabolites with no unchanged AG detectable in urine; in patients, induced metabolism to HXAG is the major metabolic pathway but >50% of AG is excreted unchanged in the urine (9, 17, 18). This represents a major species difference in metabolism.

The present study, therefore, supports the use of this model, but an observed effect does not predict whether the drug will be effective in pre- or postmenopausal women.

Apart from occasional species differences in metabolism major drawbacks to this model are: (a) it does not have the metastasizing ability of breast cancer except when it is passaged, when it loses hormone responsiveness (19); (b) our studies indicate that the rate of tumor induction in rats ovariectomized within 3 weeks of the last NMU injection is low and this would make it difficult to develop a model for postmenopausal breast cancer; (c) as demonstrated by the effect of perphenazine (Table 2), this model, as in the case of DMBA-induced mammary tumors, is sensitive to prolactin whereas it is well established that few, if any, human breast carcinomas appear to be prolactin responsive. Since estrogen stimulates rat pituitary cells to synthesize and secrete prolactin (20), the mechanism of ovariectomy may be via reduction in circulating prolactin; and (d) spontaneous regression can occur in rodent tumors and control populations of animals should be used for each batch of animals receiving test drugs.

Which other models for hormone-sensitive breast cancer may be more suitable? NMU produces mammary tumors with morphological characteristics similar to those produced by DMBA, but the DMBA model is lacking several aspects. Major deficiencies in the DMBA model are: (a) the relatively high properties of induced benign lesions compared to carcinomas (21); and (b) 25–30% of DMBA tumors do not regress on ovariectomy (22). The limitations of transplantable rat mammary models are mentioned above. One possible exception is the R3230AC tumor (23) but the ER content of this tumor is extremely low. A "hormone-dependent" tumor, termed the MXT-3590, the growth of which is stimulated by estrogens, has been described (24) but androstenedione do not inhibit tumor growth. Shaffie (25) described the growth of the breast cancer cell line MCF-7 in athymic mice and showed that tumors failed to develop in ovariectomized mice but developed in intact mice and ovariectomized mice given estrogen. It has since been shown (26) that the growth characteristics of an estrogen-responsive tumor at an estrogen-unresponsive site may mask the aggressive behavior of such a tumor. By examining the growth of a hormone-dependent tumor in a hormone-responsive site, investigators will have a model with which to better analyze the mechanisms of hormone-stimulated tumor growth.

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