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A BIOASSAY FOR THE EVALUATION OF ANTIPROLIFERATIVE POTENCIES OF PROGESTERONE ANTAGONISTS

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Summary—A bioassay which allows quantification of the antiproliferative potency of progesterone antagonists on the mammary gland was developed. For this purpose, ovariectomized rats were substituted with oestrone and progesterone and a further group simultaneously treated with the progesterone antagonists Mifepristone (=RU 38.468), Onapristone (=ZK 98.299), or ZK 112.993 (Schering AG, Berlin). A morphometric analysis of the tubulo-alveolar buds in the inguinal mammary glands revealed a dramatic antiproliferative effect of the progesterone antagonists after as little as 3 days of treatment. Several less specific mammary gland growth parameters (weight, DNA- and RNA-content) proved to be less sensitive. This bioassay measures the potency of progesterone antagonists to competitively antagonize the specific effects of progesterone on the target organ mammary gland. Further advantages of this bioassay are the use of a hormonally standardized biological system, the quantitative results, the small amount of test compound necessary, as well as the substitution with progesterone and oestrone since the antiproliferative potency of progesterone antagonists on experimental hormone dependent mammary carcinomas is most potently displayed in ovariectomized animals substituted with both sex hormones.

INTRODUCTION

A new strategy in the endocrine therapy of mammary carcinomas is the treatment with progesterone antagonists [1, 2]. The rationale of these preliminary clinical trials is based on the potent antiproliferative activity of the progesterone antagonists Mifepristone [=RU 38.486] and Onapristone [=ZK 98.299] in several progesterone receptor positive mammary tumor cells *in vivo* [3–5] and *in vitro* [6–8] as well as in progesterone receptor positive meningioma cells [9]. Nevertheless, at the moment the *in vitro* antiproliferative assays seem not to be reproducible enough to serve as a screening assay in the search of further compounds since it was recently reported that besides their antiproliferative effects *in vitro* [6–8] these progesterone antagonists could also stimulate growth of progesterone receptor positive breast cancer cells [10, 11] thus, exhibiting both antagonist and agonist-like activity depending on the cell culture conditions. Since all tumor models *in vivo* are time, work and compound intensive, there is the urgent need for a rapid and easy assay for the screening of progesterone antagonists which allows determination of their antiproliferative potencies.

In order to keep the experimental design as simple as possible it is essential to work within a hormonally standardized biological system: ovariectomized animals substituted with sex hormones should be advantageous. In view of the aim to find progesterone antagonists for the treatment of mammary carcinoma it is expedient to analyze the relevant target organ, the mammary gland. To evaluate the antiproliferative potency of progesterone antagonists quantitative data are necessary. Finally, in the screening of newly synthesized compounds it is always an essential to use a minimum of substance.

EXPERIMENTAL

Animals

Female Wistar SPF rats (Schering AG, Berlin) with 100 g body weight were kept in climatized rooms at constant temperature under controlled light conditions. Standard food (Altromin®) and water were supplied *ad libitum*.

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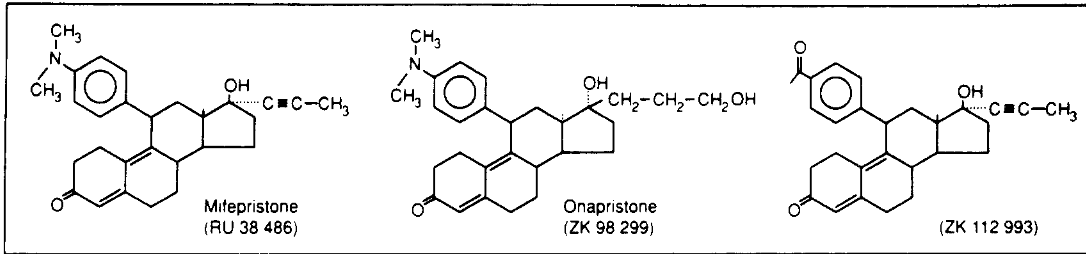

Fig. 1. Chemical structure of the progesterone antagonists Onapristone, ZK 112.993 (Schering AG, Berlin) and Mifepristone (Roussel-Uclaf).

Rats were ovariectomized under ether anaesthesia. After a period of 1 week of involution the animals were assigned randomly to treatment groups.

Experimental design

As shown in Fig. 2, 1 week after ovariectomy the rats were substituted with estrone and progesterone for 3, 4, 5 and 6 days. A dose of 10 μg estrone and 3 mg progesterone was employed, as described by Mizonobe [12]. The animals in the experimental group simultaneously received the progesterone antagonist. The inguinal mammary glands were dissected: the left prepared for morphometrical, the contralateral for biochemical analysis.

Progesterone antagonists

As progesterone antagonists Mifepristone (RU 38486, 13) Onapristone [ZK 98.299] and ZK 112.993 (Fig. 1, Schering AG, Berlin) were used [14–16] in a dose of 1 mg or 0.1 mg/animal and day.

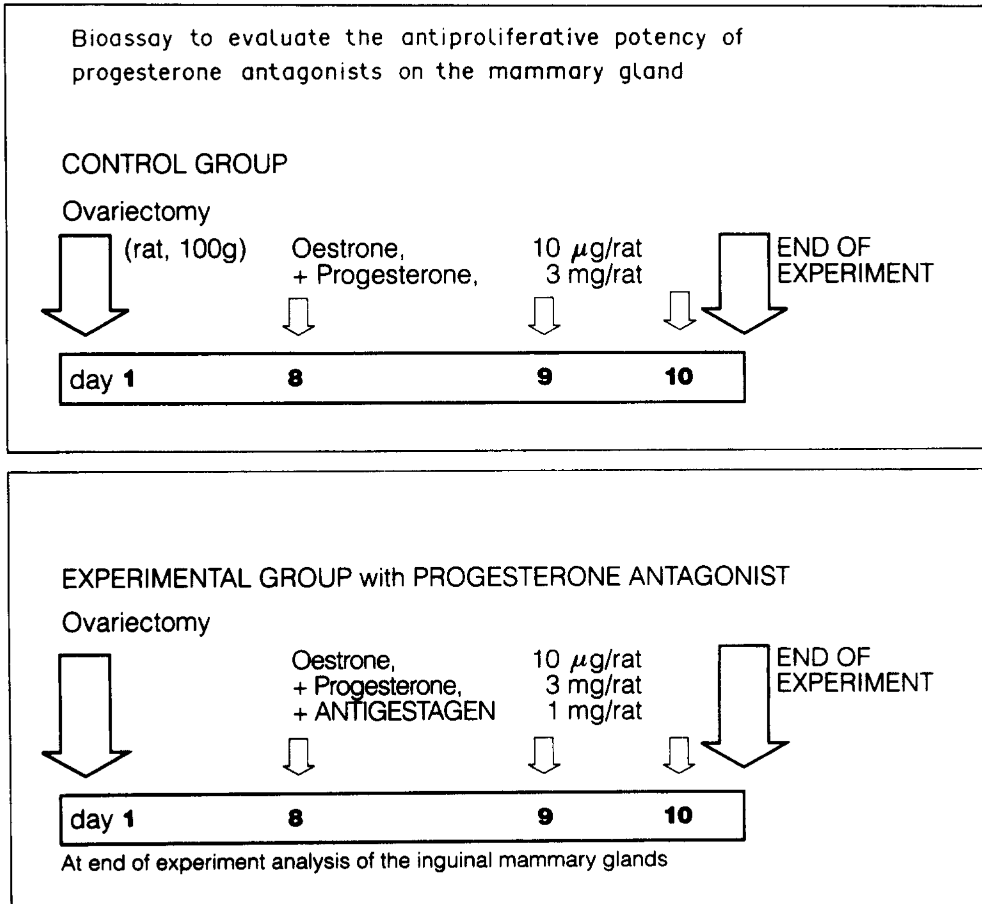

Fig. 2. Experimental design of the bioassay to evaluate the antiproliferative potency of progesterone antagonists on the mammary gland.

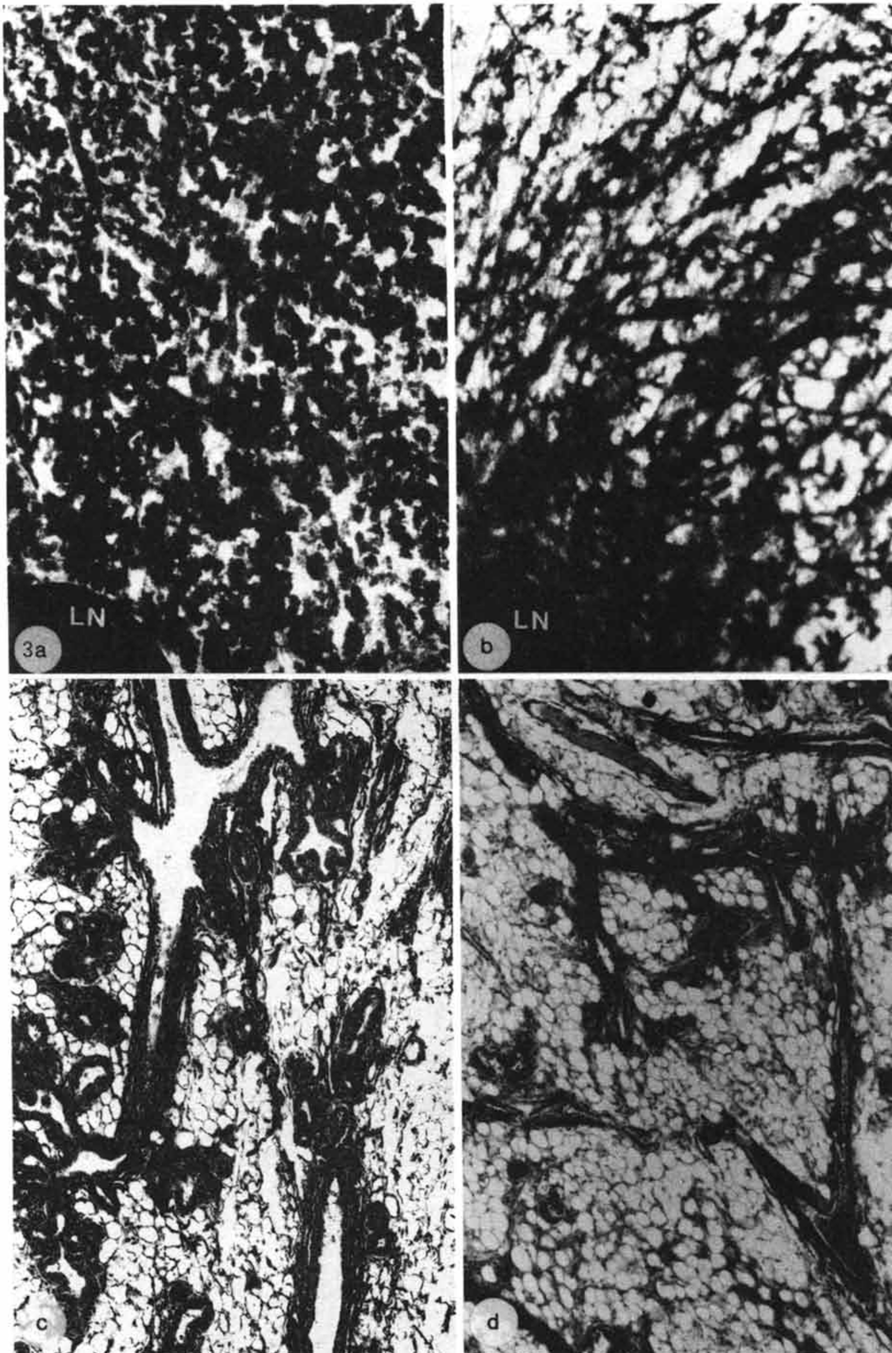

Fig. 3. Inguinal mammary glands from the control (a, c) and the experimental (b, d) group; (LN = Lymph node). **Control:** (a, c) "Whole mount preparations" (a, magnification ×11) and paraffin sections [c = Azane staining ×166] of the mammary gland were obtained from ovariectomized rats after 3 days of substitution with oestrone and progesterone. Note: pronounced development of tubulo-alveolar buds, the "ductual tree" is completely filled with end-buds. **Experimental group:** (b, d) "Whole mounts" were prepared from ovariectomized rats after 3 days of substitution with oestrone and progesterone as well as a simultaneous treatment with a progesterone antagonist [ZK 112.993]. Note: strong antiproliferative effect on the tubulo-alveolar buds. b: whole mount, c: paraffin section.

Preparation of mammary glands

The entire inguinal mammary gland was prepared for conventional paraffin sections or the so-called "whole mount technique" [17]. Glands were fixed for 24 h in Tellyesniczky's solution [18], afterwards gently removed from the skin and returned to the same solution for another 24 h. After 24 h washing in 50% ethanol the mammary glands were stained with ferric ammonium sulphate, then defatted in acetone and stored in xylene. Microphotographs were taken in transmitted light.

Morphometric analysis

The number of tubulo-alveolar buds was counted in the whole mount preparations using a 40-fold magnification. To analyze the number of end-buds, that region of the mammary gland extending from the inguinal lymph node to the body wall, which is the region of the gland containing the end-buds, was used. In the neighbourhood of the inguinal lymph node a square of 2.5 mm^2 was quantified and calculated for a tissue volume of 100 mm^3 in >10 animals.

The antiproliferative action of the progesterone antagonists on the amount of tubulo-alveolar buds is estimated for a 80% confidence interval of mean inhibition. The mean inhibition is the ratio of the mean of respective treatment group to the mean of the control group. The confidence intervals were computed using Fieller's theorem [19].

DNA and RNA analysis

DNA and RNA analysis were performed according to the method of Webb and Levy [20] with small deviations in the defatting procedure. Mammary glands were boiled in ethanol (4 h), and ether (2 h) followed by intense drying in vacuum.

RESULTS

After 3 daily injections of oestrone and progesterone, the mammary glands of ovariectomized rats displayed a pronounced development of tubulo-alveolar buds (Fig. 3a, c).

In contrast, mammary glands simultaneously treated with the progesterone antagonists displayed fewer buds (Fig. 3b, d). The wet and dry weights of mammary gland (data not shown) as well as DNA and RNA content were significantly reduced only after a longer treatment with the progesterone antagonists of 6 days (Fig. 5). Therefore the parameter tubulo-alveolar buds proved to be more sensitive to analyze the antiproliferative potency of progesterone antagonists on the mammary gland. Thus, it is possible to shorten the treatment periods in this bioassay up to 3 days.

Quantitative analysis showed that, after three successive applications of the progesterone antagonists, there was a 30–35% inhibition compared to the hormone substituted controls (Fig. 4).

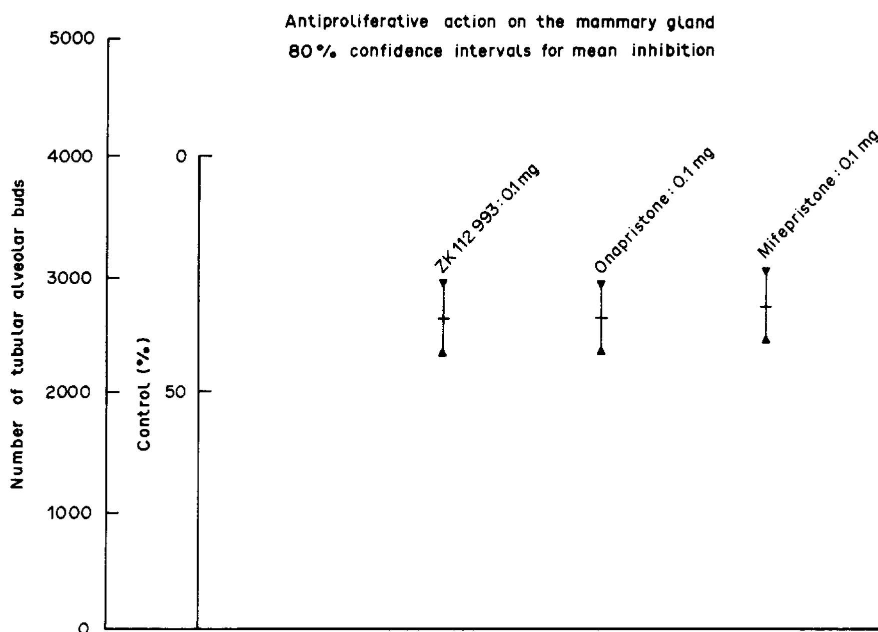

Fig. 4. Number of tubulo-alveolar buds in the inguinal mammary gland.

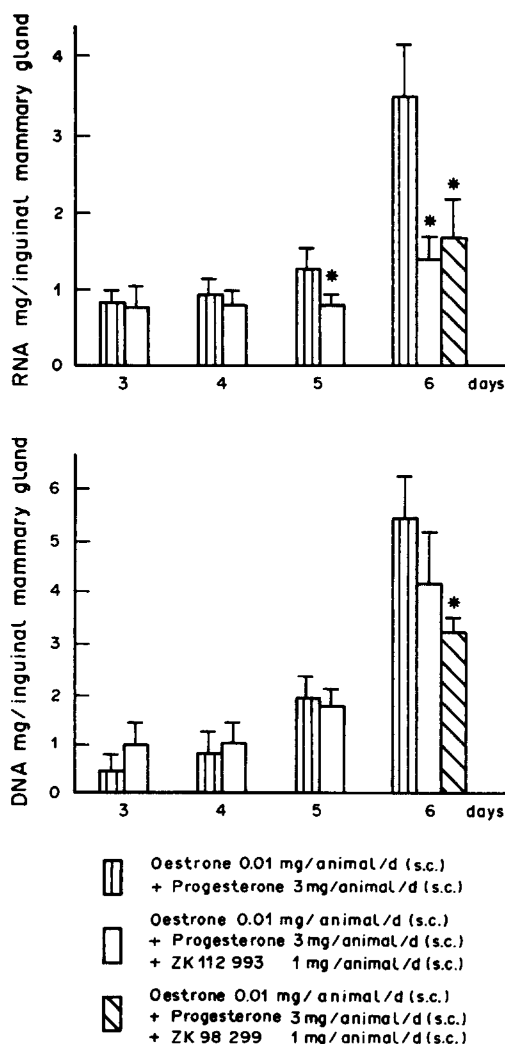

Fig. 5. Inhibition of mammary gland parameters [DNA/RNA content].

DISCUSSION

The data reveal that this bioassay allows—to our knowledge for the first time—analyses of the antiproliferative potency of progesterone antagonists on the target organ mammary gland in an easy and rapid manner. The use of morphometrical and biochemical methods permits a quantitative evaluation of the antiproliferative potency of progesterone antagonists on the tubulo-alveolar mammary gland buds. The morphometrical analysis of the number of tubulo-alveolar buds proved to be the most sensitive parameter in this screening assay and allowed a shortening of the treatment period to only 3 days using a minimal amount of test compound.

As this is a screening procedure and not a confirmatory procedure there was no Bonferroni correction (adjustment of α to correct for the number of tests conducted) [21] which would cause the significance level to depend on the number of substances tested in the experiment. This would be most undesirable for a screening procedure. Furthermore, we have choosen an 80% confidence interval so as not to summarily dismiss substances which are of potential interest. No Bonferroni correction means the power computations are done with $\alpha = 0.20$ and a t-test. In addition, we have unpublished data on several experiments with different numbers of animals per treatment group: with as few as 5 animals, there is an adaquate power to demonstrate statistically significant effects of these test compounds on glandular buds.

The antiproliferative potency of progesterone antagonists in this bioassay depend on the inhibition of the well-known stimulating effect of progesterone on the development of mammary gland buds [22, 23]. Thus, it can be concluded that this bioassay measures the potency of a compound to competitively antagonize the effects of progesterone [24, 25] at the receptor level in the target organ, the mammary gland and also in the target cells, the end-bud epithelial cells. The functional differentiation of the epithelial cells induced by progesterone after a priming effect of estrone explains the sensitivity of the morphometry of the epithelial end-buds in contrast to the poor sensitivity of the biochemical procedure used analyzing in addition to epithelial cells all the further cellular components of the mammary gland, the fat cells and the fibroblasts, which do not sufficiently respond to progesterone and estrogens in a functional manner [23–25].

For the measurement of the antiproliferative potency of progesterone antagonists it is essential to substitute the animals with progesterone and estrogen since the stimulating effect of progesterone on the mammary gland buds is only induced when both hormones are given [24, 25, 27–29]. Therefore, it fits that antiproliferative effects of progesterone antagonists could only be detected after substitution with both sex hormones (data not shown). The fact that the differentiating potency of progesterone on the epithelial cells also necessitates the priming effect of estrogens [25, 27] already

theoretically implies that antioestrogens should also be able to disturb the differentiating potency of progesterone by blocking the priming effect of estrogen. This was indeed detected for some antioestrogens (unpublished data). It fits that all of the tested progesterone antagonists are—unlike some antiestrogens—not able to induce antiestrogenic properties in this mammary gland assay and do not block the induction of the estrogen dependent glandular ducts (unpublished data). Nevertheless, some progesterone antagonists could be able to enfold antiuterotrophic effects [30, 31] although these compounds are known to have no affinity to the estrogen receptor [16]. Therefore, looking at uterine weights in this bioassay does not strengthen the power of the test system.

Finally, the substitution with oestrone and progesterone in this bioassay deals with the fact that the antiproliferative potency of progesterone antagonists on experimental hormone dependent mammary carcinomas is most potently displayed in ovariectomized animals substituted with both sex hormones [30]. So far there is a strong correlation of the antiproliferative potency of these progesterone antagonists in this bioassay and in progesterone receptor positive experimental mammary carcinomas [4, 5, 30].

Theoretically, it should be possible—and this is our future goal—by modifying the assay design and replacing progesterone with an uncharacterized compound to establish a potential bioassay to evaluate progestational activity for the first time on the mammary gland as a target organ which would be comparable to the classical [32] and various new [33] bioassay systems on the uterus as the target organ.

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