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Original Paper

Androgen-like and Anti-androgen-like Effects of Antiprogestins in Human Mammary Cancer Cells

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In addition to their antiprogestational activity, the antiprogestins RU486, ZK98.299 and ZK98.734 possess varying antiglucocorticoid as well as androgen-like or antiandrogen-like properties in human mammary cancer cells. The human mammary cancer cell line MFM-223, which contains only androgen receptors, was used as a model to investigate androgen receptor mediated effects of these antiprogestins. Proliferation of MFM-223 cells is inhibited by androgens and does not respond to oestrogens, progestins and glucocorticoids. As shown in proliferation assays, ZK98.734 was a strong inhibitor of cell proliferation. This effect was antagonised by the antiandrogen hydroxyflutamide. ZK98.734 was found to displace [^{3}H]R1881 from the androgen receptor in MFM-223 cells, substantiating the involvement of the androgen receptor. The antiprogestin ZK98.299 failed to influence the proliferation of MFM-223 cells. ZK98.299 did not bind to the androgen receptor and was devoid of androgenic or antiandrogenic activity. RU486 bound to the androgen receptor. It was a weak inhibitor of MFM-223 cell proliferation, but the inhibition of proliferation by RU486 was not antagonised by hydroxyflutamide. This effect was probably not mediated by the androgen receptor. RU486 had antiandrogenic activity in this cell line, as it antagonised the inhibitory effect of dihydrotestosterone at a 100-molar excess. These results were confirmed by transfection experiments with an MMTV-*CAT* construct in the same cell line, demonstrating the biological function of the ZK98.734–androgen receptor complex. ZK98.299 and RU486 were not able to induce CAT activity. The different androgenic or antiandrogenic properties of the antiprogestins investigated should be considered when selecting antiprogestational compounds for clinical applications, as a partial androgenic activity may be of benefit in breast cancer but can have undesired side-effects in other diseases.

Key words: androgen, androgen receptor, antiandrogen, antiprogestin, breast cancer, mammary cancer cells

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INTRODUCTION

ANTIPROGESTINS REPRESENT a new class of antisteroidal agents, offering new horizons in the treatment of endometriosis, leiomyomata of the uterus, meningioma, and in different fields of birth control [1]. Preliminary studies have reported on the clinical use of the antiprogestin RU486 in the treatment of disseminated breast cancer [2]. Antiprogestins block the effect of progestagenic agents by competitive displacement of progestins from the progesterone receptor. Additionally, antiprogestins can bind to the glucocorticoid and/or the androgen receptor with high affinity, but they are devoid of binding to the oestrogen receptor [3]. However, in the rodent uterus, some oestrogenic effects of ZK98.299 have recently been reported [4]. The significance of this multi-receptor binding for the clinical effects of antiprogestins is not yet fully understood.

Breast cancer growth is thought to be regulated by a variety of steroid hormones. Stimulation of proliferation by 17β-oestradiol was observed in several human mammary cancer cell lines [5, 6]. The *in vitro* role of progestins, which are used in the treatment of metastasising breast cancer [7], is less well understood [8]. Some studies have reported the stimulation of proliferation of mammary cancer cells [8–10], whereas other authors have found inhibitory effects [11–13] or no

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effect of progestins [14]. Low concentrations of the androgen dihydrotestosterone are ineffective in MCF-7 or EFM-19 cells, but inhibit the proliferation of ZR-75-1 [15] and MFM-223 cells [16]. This demonstrates the potentially inhibitory role of androgens. Glucocorticoids stimulate the proliferation of EFM-19 [17] and inhibit MCF-7 cells [14].

Steroids, which bind to several receptors, can exert biological effects via two or three steroid hormone receptors. Medroxyprogesterone acetate, which is widely used in the palliative treatment of disseminated breast cancer, binds to the progesterone, glucocorticoid and androgen receptor. All three receptors are synergistically involved in the inhibition of breast cancer growth by this compound [15, 18]. Androst-5-ene-3β, 17β-diol, a metabolite of dehydroepiandrosterone, is another example of this type of steroid. It stimulates the proliferation of MCF-7 but inhibits MFM-223 cells [19]. This steroid can thus exert opposite effects on mammary cancer cells via different steroid hormone receptors.

Antiprogestins bind to the progesterone, glucocorticoid and androgen receptor. They can possibly exert complex biological effects in mammary cancer cells, which may be mediated by several classes of steroid hormone receptors. In the present study, MFM-223 cells were used to evaluate androgen receptor mediated effects of the antiprogestins RU486, ZK98.299 and ZK98.734. The growth of MFM-223 cells is inhibited by androgens but does not respond to oestrogens, progestins and glucocorticoids [18]. This cell line, therefore, represents a good *in vitro* model for the investigation of androgen receptor mediated effects in human mammary cancer cells without interference with other steroid hormone receptors.

MATERIALS AND METHODS

Cell culture and media

MFM-223 cells were originally derived from the pleural exudate of a breast cancer patient. MFM-223 cells are characterised by their high level of androgen receptors in combination with low levels of those for oestrogens, progestins and glucocorticoids. Progesterone receptors are not induced by incubation with oestrogens. Proliferation of MFM-223 cells is strongly inhibited by androgens, but not influenced by oestrogens, progestins and glucocorticoids [16]. This cell line is therefore well suited for the investigation of androgen receptor mediated effects without interference with other steroid hormone receptors. Standard growth medium was based on Eagle's minimum essential medium and was enriched with 4 mM L-glutamine, 67 mg/l gentamicin sulphate (all from Biochrom, Berlin, Germany), 2.5 mg/l transferrin (Serva, Heidelberg, Germany), 40 IU/l insulin (Hoechst, Frankfurt, Germany) and 10% fetal bovine serum (Boehringer-Mannheim, Germany).

Steroid hormone receptor assays

These assays were performed as previously described [18, 20]. Briefly, the cells were grown until near-confluence. The cultures were washed twice with PBS, before [^{3}H]-labelled R1881 (methyltrienolone, NEN, Frankfurt/ Dreieichenhain, Germany) and increasing concentrations of the antiprogestins were added. [^{3}H]R1881 was used as high affinity androgen receptor ligand. Binding of unlabelled antiprogestins was monitored by the partial displacement of [^{3}H]R1881 from the androgen receptor. The glucocorticoid receptor was blocked by the simultaneous application of triamcinolone acetonide. After incubating the cells with ligands for 4 h at 37°C, the cultures were washed twice with PBS. Then 0.5 ml of 1 M NaOH was added for cell lysis. After overnight incubation, the samples were neutralised with 1 M HCl. Finally, aliquots were counted in a β-scintillation counter.

Proliferation assays

Cells were seeded in multiple four-well cluster dishes (2 cm^2) (Nunc, Roskilde, Denmark) at a density of 10^4 or 1.5×10^4 cells per well. After a 1-day attachment phase, the medium was replaced by fresh culture medium and the hormones were added. Quadruplicate cultures were withdrawn, trypsinised and counted in a haemocytometer. Proliferation assays with MFM-223 cells were performed in standard growth medium, as MFM-223 cells do not proliferate well in DCM and do not respond to oestrogens. The following steroids were used in the experiments: the androgen dihydrotestosterone (DHT), the antiandrogen hydroxyflutamide (HF) (Essex, Munich, Germany), the glucocorticoid dexamethasone (dex) (Sigma, Munich, Germany), the progestin R5020 (promegestone), and the antiprogestins RU486 (11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(1-propinyl)-4,9(10)-estradien-3-one), ZK98.299 (11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(3-hydroxy-prop-1(Z)-enyl)-4,9(10)-estradien-3-one) and ZK98.734 (11β-(4-dimethylaminophenyl)-17α-hydroxy-17β-(3-hydroxypropyl)-13α-methyl-4,9-gonadien-3-one) (Schering, Berlin, Germany).

Transfection experiments

In the transfection experiments, an artificial gene construct was introduced into viable cells. This gene construct contains a hormone responsive promotor (MC 3-1 CAT) [21]. If the cells possess the corresponding hormone receptor and activating hormones are added, the promotor initiates transcription of the reporter gene (*CAT*, chloramphenicol acetyl transferase), which is also located on the transfected gene construct. The transfected cells translate the resulting mRNA into the protein, which can easily be monitored by an enzyme assay (CAT-assay). The hormone responsive element of the promoter used is responsive to hormone-activated androgen-progesterone and glucocorticoid receptors. As MFM-223 lack sufficient concentrations of progesterone and glucocorticoid receptors, only androgens can induce *CAT*-activity in this model system.

The experiments were performed as previously described [22]. In short, cells were plated out at a concentration of 1.5×10^6 cells per 60-mm cell culture dish. On the next day, a mixture of supercoiled DNA including 4 μg chloramphenicol acetyltransferase (*CAT*) construct, either MC 3-1 CAT or RSV CAT and 2 μg RSV luciferase [21] was introduced to each plate by the calcium phosphate method. 20 h post transfection, media were changed to medium containing 5% stripped fetal bovine serum and the steroids under investigation were added. After 48 h, the cells were collected and lysed by three cycles of freezing and thawing in 100 μl of a modified Tris-buffer [23]. Cytosolic extracts were tested for CAT- and luciferase-activity. The luciferase assay demonstrated the uniformity of transfection efficiency (data not shown).

RESULTS

Binding of antiprogestins to the androgen receptor in MFM-223 cells

Using MFM-223 cells, the binding of the antiprogestins under investigation to the androgen receptor was investigated in competition experiments with [^{3}H]R1881 (Figure 1). The competition curves demonstrate that DHT binds to the androgen receptor with high affinity. RU486 and ZK98.734 also effectively displaced [^{3}H]R1881 from the androgen receptor at concentrations above 100 nM, demonstrating an equivalent affinity of these two compounds to the androgen receptor. Binding of ZK98.299 was found only at the very high concentration of 10 μM.

Inhibition of proliferation of MFM-223 cells by antiprogestins

Inhibitory effects of 1 μM concentrations of the antiprogestins RU486, ZK98.299 and ZK98.734 were tested during a 10-day incubation period. ZK98.299 had no inhibitory or stimulatory effects on the proliferation of MFM-223 cells. ZK98.734 strongly inhibited the proliferation of this androgen receptor positive cell line. RU486 showed an intermediate activity and reduced tumour cell proliferation only slightly (Figure 2).

The inhibitory effect of ZK98.734 was dose-dependent during a 7-day incubation period. A reduction of cell number to less than 50% of the control was observed at concentrations of 100 nM and more (Figure 3). The antiandrogen hydroxyflutamide was used to antagonise the inhibition by ZK98.734. Hydroxyflutamide was ineffective at equimolar concentrations, but 1 μM hydroxyflutamide was able to antagonise the effect of up to 100 nM ZK98.734. These data demonstrate that the effect of ZK98.734 is mediated by the androgen receptor.

The antiprogestin RU486 also inhibited the proliferation of MFM-223 cells, but hydroxyflutamide did not antagonise the effect of RU486 (Figure 4). RU486 binds to the progesterone, androgen and glucocorticoid receptor, possessing antiproges-

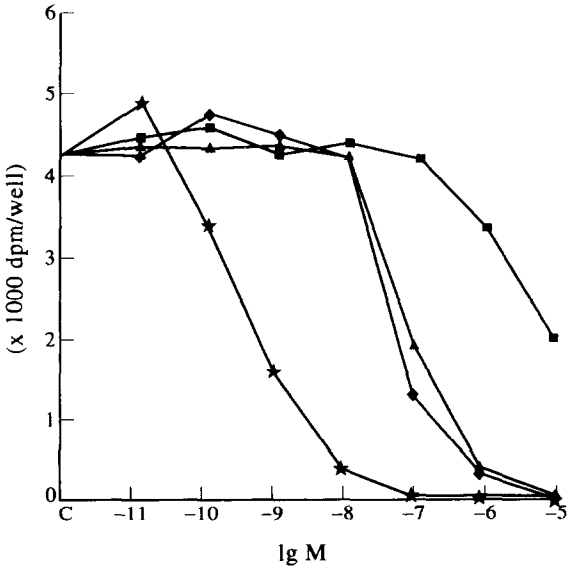

Figure 1. Competitive displacement of 0.5 nM [^{3}H]R1881 from the androgen receptor by increasing concentrations of dihydrotestosterone (DHT) (★), RU486 (▲), ZK98.734 (◆) and ZK98.299 (■). C is the control value. Each point represents a duplicate sample.

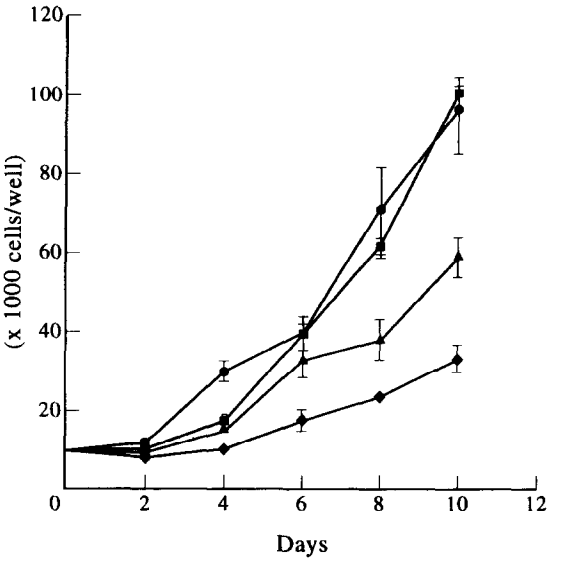

Figure 2. Proliferation of MFM-223 cells during incubation with 1 μM of RU486 (▲), ZK98.299 (■) and ZK98.734 (◆). Untreated control cultures (●). All values are means of quadruplicate cultures ± S.D. If no S.D. bars are shown, these values lie within the symbol.

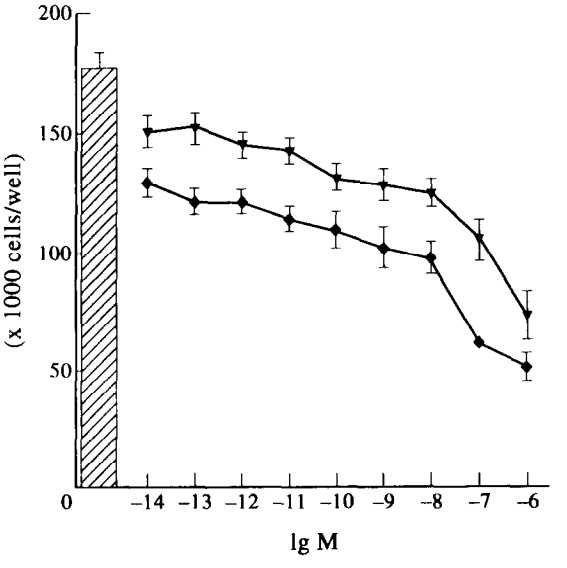

Figure 3. Dose-dependent inhibition of cell proliferation by ZK98.734 (◆) and antagonisation of the inhibitory effect of ZK98.734 by the simultaneous application of 1 μM hydroxyflutamide (▼) in comparison with the untreated controls (bar). All values are means of quadruplicate cultures ± S.D. If no S.D. bars are shown, these values lie within the symbol.

tational and antiglucocorticoidal properties. The interference of these steroids with the inhibitory potential of RU486 was therefore tested. The progestin R5020 and the glucocorticoid dexamethasone did not interfere with the inhibition of proliferation by RU486 (Table 1). These results suggest that additional mechanisms are involved in the action of RU486.

Alternatively, RU486 had antiandrogenic properties in MFM-223 cells. Proliferation of MFM-223 cells was inhibited by DHT in the concentration range of 0.01 pM up to 10 nM, demonstrating the strong inhibitory potential of DHT in this cell line (Figure 5). The simultaneous application of 1 μM

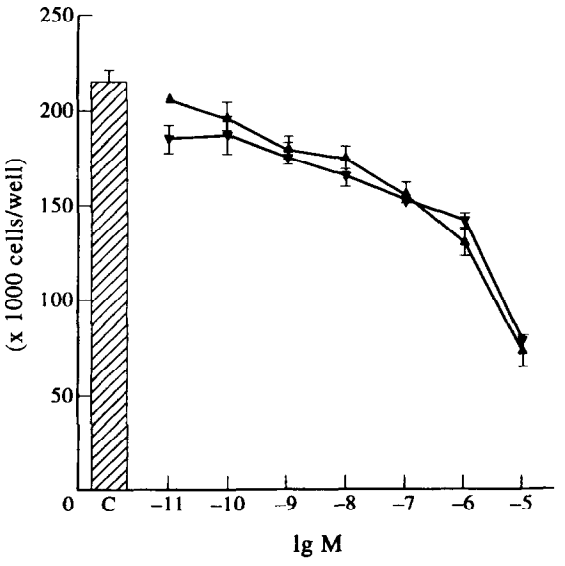

Figure 4. Dose-dependent inhibition of cell proliferation by RU486 (▲) and lack of antagonisation during simultaneous application of 1 μM hydroxyflutamide (▼) in comparison with the untreated controls (bar). All values are means of quadruplicate cultures ± S.D. If no S.D. bars are shown, these values lie within the symbol.

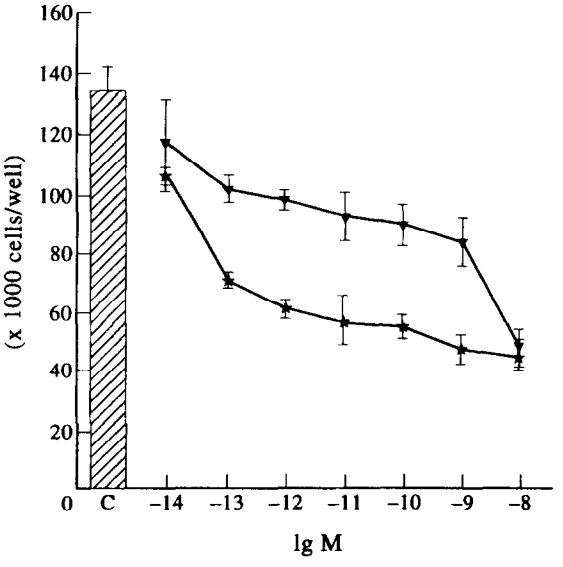

Figure 5. Antagonisation of the inhibitory effect of dihydrotestosterone (DHT) (★) by the simultaneous application of 1 μM RU486 (▼) in comparison with the untreated control cultures (bar). All values are means of quadruplicate cultures ± S.D. If no S.D. bars are shown, these values lie within the symbol.

Table 1. *Inhibition of cell proliferation of MFM-223 cells by RU486 and effects of potentially interfering hormones*

	RU486 alone	+ HF	+ R5020	+ Dex	+ DHT
RU486 1 μM	74.3 ±3.3	78.3 ±2.1	77.3 ±5.0	77.3 ±1.7	36.7 ±7.7
RU486 100 nM	75.7 ±15.2	83.3 ±3.1	76.0 ±2.9	75.0 ±6.5	34.3 ±5.4

HF, hydroxyflutamide; Dex, dexamethasone; DHT, dihydrotestosterone. All values were derived from three experiments with quadruplicate cultures in each experiment and are expressed as percentages of the untreated controls (158 500 cells per well). HF, R5020 and Dex were applied at a concentration of 1 μM.

RU486 antagonised the inhibition of proliferation by DHT, demonstrating antiandrogenic properties of RU486.

Transfection experiments

In the transfection experiments, an artificial DNA plasmid was introduced into the cells. This DNA contains parts of the hormone-dependent MMTV-promoter and the *CAT*-gene, which is used as reporter gene. If an androgen agonistic compound binds to the androgen receptor present in MFM-223 cells, the MMTV-promotor is activated, and the *CAT*-gene will be expressed, which can be monitored in the CAT-assay.

After introducing the hormone-dependent DNA-construct into MFM-223 cells, the androgen DHT induced the expression of *CAT*-activity (Figure 6). No significant *CAT*-activity was observed in the absence of DHT. The glucocorticoid dexamethasone and the progestin R5020 both showed no activity. The lack of activity with Dex is probably due to the low level of glucocorticoid receptors present in MFM-223

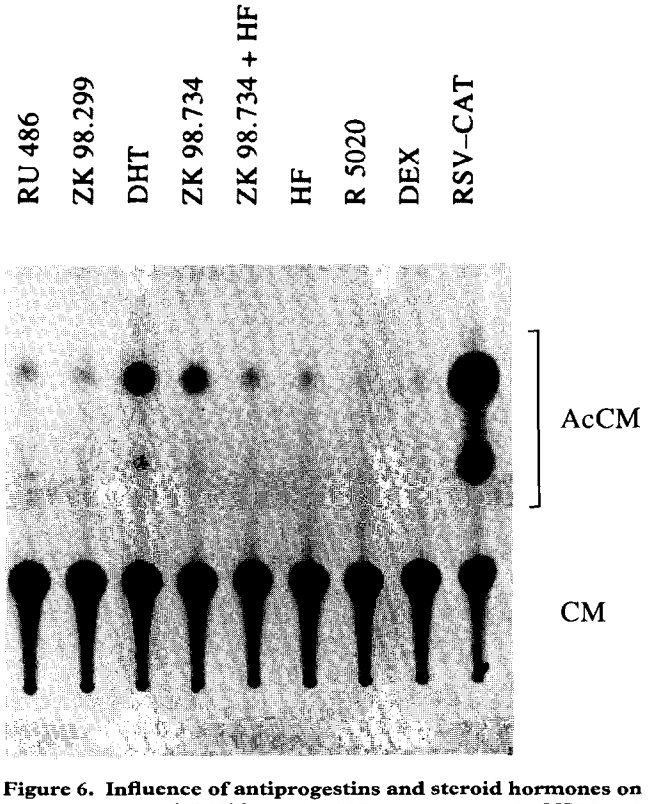

Figure 6. Influence of antiprogestins and steroid hormones on the expression of *CAT* from the MC3-1 promotor in MFM-223 cells. The RSV-*CAT* construct served as positive control. Only DHT and the antiprogestin ZK98.734 were able to induce *CAT*-activity. The mobilities of the substrate chloramphenicol (CM) and the acetylated forms (AcCM) are indicated on the right.

cells. Among the antiprogestins tested, RU486 and ZK98.299 did not induce *CAT*-activity in MFM-223 cells. Significant *CAT*-activity was only found after application of the antiprogestin ZK98.734. This effect of ZK98.734 was antagonised by the simultaneous application of the antiandrogen hydroxyflutamide, supporting the involvement of the androgen receptor.

DISCUSSION

The humam mammary cancer cell line MFM-223 was used to evaluate androgen-like and antiandrogen-like properties of the antiprogestins RU486, ZK98.299 and ZK98.734. RU486 possesses antiandrogenic properties in this model system, whereas ZK98.734 acts like an androgen. ZK98.299 does not interact with the androgen receptor. We used proliferation, binding and transfection experiments, and corresponding results were gained by these methods. MFM-223 cells represent the only androgen responsive mammary cancer cell line, which is insensitive to other steroid hormones and the study was therefore restricted to this cell line.

ZK98.734 inhibits the proliferation of MFM-223 cells. The antagonistic effect of hydroxyflutamide, the binding studies to the androgen receptor and the transfection experiments demonstrated the involvement of the androgen receptor. ZK98.299 showed no androgen-like activity in all the experiments performed.

Binding of RU486 to the androgen receptor was comparable to the binding of ZK98.734. However, RU486 antagonised the inhibitory effect of DHT. The dose–response curve of DHT with RU486 revealed that RU486 has antiandrogenic activity if applied at least at a 100-molar excess (10 nM DHT plus 1000 nM RU486), corresponding to the binding affinities of both compounds. Antiandrogenic activity of RU486 has also been found in other systems [24]. Alternatively, RU486 inhibits the proliferation of MFM-223 cells in the absence of DHT. The inhibition of proliferation by RU486 can not be antagonised by the antiandrogen hydroxyflutamide. RU486 is also ineffective in the transfection experiments. Therefore, it is unlikely that the inhibitory activity of RU486 is mediated by the androgen receptor. RU486 inhibits the proliferation of T47D and MCF-7 mammary cancer cells [25, 26]. RU486 appears to be cytotoxic in these cell lines, while the progesterone agonist R5020 exerts only cytostatic effects [27]. The antiproliferative potential of RU486 seems to be mediated through the progesterone receptor in MCF-7 and T47D cells. It has been speculated that the binding of RU486 to the progesterone receptor induces an altered confirmation or activation of the progesterone receptor leading to inappropriate recognition of genomic sites [28]. In MFM-223 cells, containing only very low levels of progesterone receptors, the involvement of the progesterone receptor is not likely, as neither R5020 nor the antiprogestin ZK98.299 are active in this cell line (see above). The mechanism underlying the inhibition of proliferation by RU486 in MFM-223 cells is not yet clear.

The antiprogestins under investigation also exhibited different antiglucocorticoid potentials. They possessed different androgenic or antiandrogenic activities in MFM-223 mammary cancer cells. RU486, in relation to its antiprogestational activity, was the most active antiglucocorticoid compound, while ZK98.734 was the least active one. ZK98.299 had an intermediate effect [3]. The binding of antiprogestins to the progesterone, androgen and glucocorticoid receptor in combination with the presence of these steroid hormone receptors in mammary cancer cells results in a very complex pattern of biological effects of antiprogestins in human breast cancer. The effects of antiprogestins may also depend on the type of tissue tested and the exact experimental conditions used [29, 30]. It has been the aim of this study to reveal the involvement of the androgen receptor in this process, and the results should help to select appropriate antiprogestins for special clinical applications.

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