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The antiprogestins mifepristone and onapristone reduce cell proliferation in the canine mammary carcinoma cell line CMT-U27

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Summary. Canine mammary tumours (CMTs) represent nearly half of all tumours in female dogs and some 50% have malignant behaviour. Simple epithelial carcinomas have shorter disease free periods after surgery and a higher reduction of the proliferation index reduction after antiprogestin aglepristone treatment *in vivo* related to the expression of progesterone receptors (PR). These findings make simple carcinomas good candidates for endocrine therapy. To further explore this possibility, the effects of the antiprogestins mifepristone (RU486) and onapristone (ZK299) on cell viability and PR expression of the canine mammary carcinoma cell line isolated from a *simple epithelial* carcinoma CMT-U27 were studied. Twenty five percent of CMT-U27 control cells expressed PR. RU486 ($p<0.05$) and ZK299 ($p<0.05$) reduced the number of viable cells (WST-8 test) at 24h but only the latter treatment reduced significantly PR expression in viable tumour cells at 24h of incubation. The results suggest that both RU486 and ZK299 induce a decrease in the number of viable CMT-U27 tumour cells with different effects on PR expression. The canine mammary carcinoma cell line CMT-U27 is sensitive to the effects of antiprogestins and may serve to further explore the role of these drugs in canine mammary carcinomas.

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Introduction

Canine mammary tumours (CMTs) represent nearly half of all tumours in female dogs and some 50% have malignant behaviour (Sorenmo et al., 2013). Epidemiologic, clinical and experimental data indicate that CMTs are hormone-dependent, that is, are strongly influenced by ovarian hormones, mainly estrogens and progesterone (P) (Sorenmo et al., 2013). Prolonged exposure to high concentrations of progesterone during the comparatively long luteal phase of the oestrous cycle is suspected to be the key event in canine mammary tumorigenesis (Evans et al., 1969). Ovariectomized female dogs when exposed to progestins for 6-12 months developed mammary gland hyperplasias and/or benign tumours (Selman et al., 1994; Bhatti et al., 2007). Further, the occurrence of mammary carcinomas has been reported in dogs upon administration of high doses of progestins (Kwapien et al., 1980). Studies of gene expression profiles indicate a strong cell proliferation inducing effect of progestins in canine mammary hyperplasia and carcinoma (Rao et al., 2009). In women with breast cancer, hormone therapy is mostly focused on the blockade of oestrogen receptors as well as on the use of aromatase inhibitors, because these receptors are expressed in approximately 70% of all cases (Knoop and Rasmussen, 2007; Lanari et al., 2012). In the dog, two-thirds of mammary carcinomas express P receptors (PR) (Geraldes et al., 2000; Martín de las Mulas et al., 2005;

Chang et al., 2009) with differences in expression level among different histological subtypes. Thus, luminal epithelial cell-type or *simple epithelial* carcinomas (Misdorp et al., 1999) have lower PR expression than tumours composed of luminal epithelial and myoepithelial cells, either alone or combined with mesenchymal components (the so-called *complex* and *mixed* carcinomas, respectively) (Misdorp et al., 1999; Martín de las Mulas et al., 2005). *Simple epithelial* carcinomas have shorter disease free periods after surgery than their *complex* and *mixed* counterparts (Martín de las Mulas et al., 2005) and may thus be the histological subtype that benefits most from the use of endocrine therapy. Recently, neoadjuvant administration of the antiprogestin aglepristone (RU534) to non-spayed female dogs with spontaneous mammary carcinomas was shown to reduce tumour cell proliferation on a PR-expression basis (Guil-Luna et al., 2011). In that work, the highest decrease in cell proliferation was observed in *simple epithelial* carcinomas. The aim of the present study was to analyze the effects of the RU534 analogue mifepristone (RU486) and the antiprogestin onapristone (ZK299) on a PR-positive canine mammary carcinoma cell line isolated from a *simple epithelial* carcinoma.

Materials and methods

Cell line

The CMT-U27 cell line was established from a canine simple mammary carcinoma and has been given an account previously (Hellmén, 1992; Misdorp et al., 1999; Król et al., 2009, 2010; Pawlowski et al., 2011). RU486 and ZK299 were kindly provided by Exelgyn, Paris (France) and Shering, Berlin (Germany), respectively.

Cell culture

The cell line was cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA), supplemented with 10% foetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), penicillin-streptomycin (50 iU mL^{-1}) and kept at 37°C in 5% CO_2 atmosphere in 75 cm^2 culture flasks to 80% confluence. For the experiments, cells were grown in phenol red-free and steroid-deficient RPMI 1640 medium with antibiotics but without FBS supplemented.

Treatment of cells

Initially, the cells were cultured in complete RPMI medium without and with 0.01% ethanol for 24, 48 hours respectively. No significant differences in cell number were observed. Thus, the experiments were carried out with 0.01% ethanol supplement. CMT-U27 cells were treated with RU486 and ZK299 in absolute ethanol (final concentration 0.01%) at 10^{-2} M, 10^{-3} M , 10^{-4} M and 10^{-6} M during 24 h and 48 h each. Control cells were treated with 0.01% ethanol for 24 and 48 hours. All experiments were performed in triplicate.

Cell proliferation and viability assay

The colorimetric WST-8 assay (Promokine, Sweden) based on the WST-8 reduction by cellular dehydrogenases to an orange formazan product which is directly proportional to the number of living cells was used. Firstly, following the manufacturer's recommendations, a calibration curve using a known number of cells was performed (Fig. 1). Then, different concentrations of cells were tested in a range of 1250 to 20000 cells/well in the different incubation times and the growth rate for each range (initial number of cells/final number of cells) was calculated. The optimal number of cells per well ensuring exponential cell growth for the entire period of the assay was first established at 2500 cells/well (Fig. 1). Then, cells were seeded in 96-well plates at a density of 2500 cells per well. After 24 h attachment and three times rinsing in phosphate buffered saline, the medium was changed for phenol-red free steroid-deficient RPMI 1640 medium and the drugs (RU486 and ZK299) were added. After incubations, 10 μl of CCVK-I solution was added and measures of the absorbance at 450 nm were performed using a Multiscan Ascent microplate reader. Each sample was analysed in triplicate, and the mean value of absorbance was used as the final result.

PR expression immunocytochemical assay

Cultured cells were fixed in 4% formalin and embedded in paraffin using cell culture block technique (Li et al., 2005; Andersson et al., 2006; Mote et al., 2001). The monoclonal mouse PR antibody (clone 10A9) isotype IgG_2 (Immunotech, Marseille, France) diluted 1:400 was used for the detection of PR expression. A commercial diluent (Dako, Barcelona, Spain) was used. Heat induced antigen retrieval in a water bath at 95-99°C with 0.01 M citrate buffer (pH 6.0) for 25 min was used. After cooling down at room temperature for about 30 min, slides were covered with 10% normal goat serum in PBS for 30 min before incubation with the primary antibody for 18 hours at 4°C. The avidin-biotin-peroxidase complex (Vector Laboratories; Burlingame, CA, USA) was applied for 1 hour at room temperature. The chromogen, 3,3-diaminobenzidine tetra-hydrochloride (Sigma, Saint Louis, USA) diluted 0.035% in 0.05 M Tris containing 0.3% of hydrogen peroxide was applied to the slides for 1 min at 20-22°C. For negative controls, the primary antibody was replaced by mouse IgG_2 (Dako, Barcelona, Spain). As positive control, formalin-fixed, paraffin embedded tissue samples of canine normal mammary gland were used.

The number of positive and negative cells was counted at a magnification of 40x by two different pathologists to ensure uniformity. All cells present in

each slide (5 slides for each with an average of 1500 cells/slide) were counted. PR expression was expressed as the percentage of positive cells related to the total number of cells. Further, staining intensity was scored on a scale of 0 to 3+ and defined as follows: 0, no staining; 1+, weak; 2+, intermediate and 3+, strong staining.

Statistical analysis

Statistical analysis was carried out by GraphPad Software 3.05 (San Diego, CA). The values were evaluated for approximate normality of distribution by the Kolmogorov-Smirnov test. Differences between treatments were assessed by Mann-Whitney test. Results were expressed as mean ± SD. A $p<0.05$ was regarded as statistically significant.

Results

Cell proliferation and viability

Concerning proliferation assay, a start concentration of 2500 cells per well showed a characteristic growth pattern of cultured cells (Fig. 1) and adequate for the viability assay. It was observed that the first 48h (the

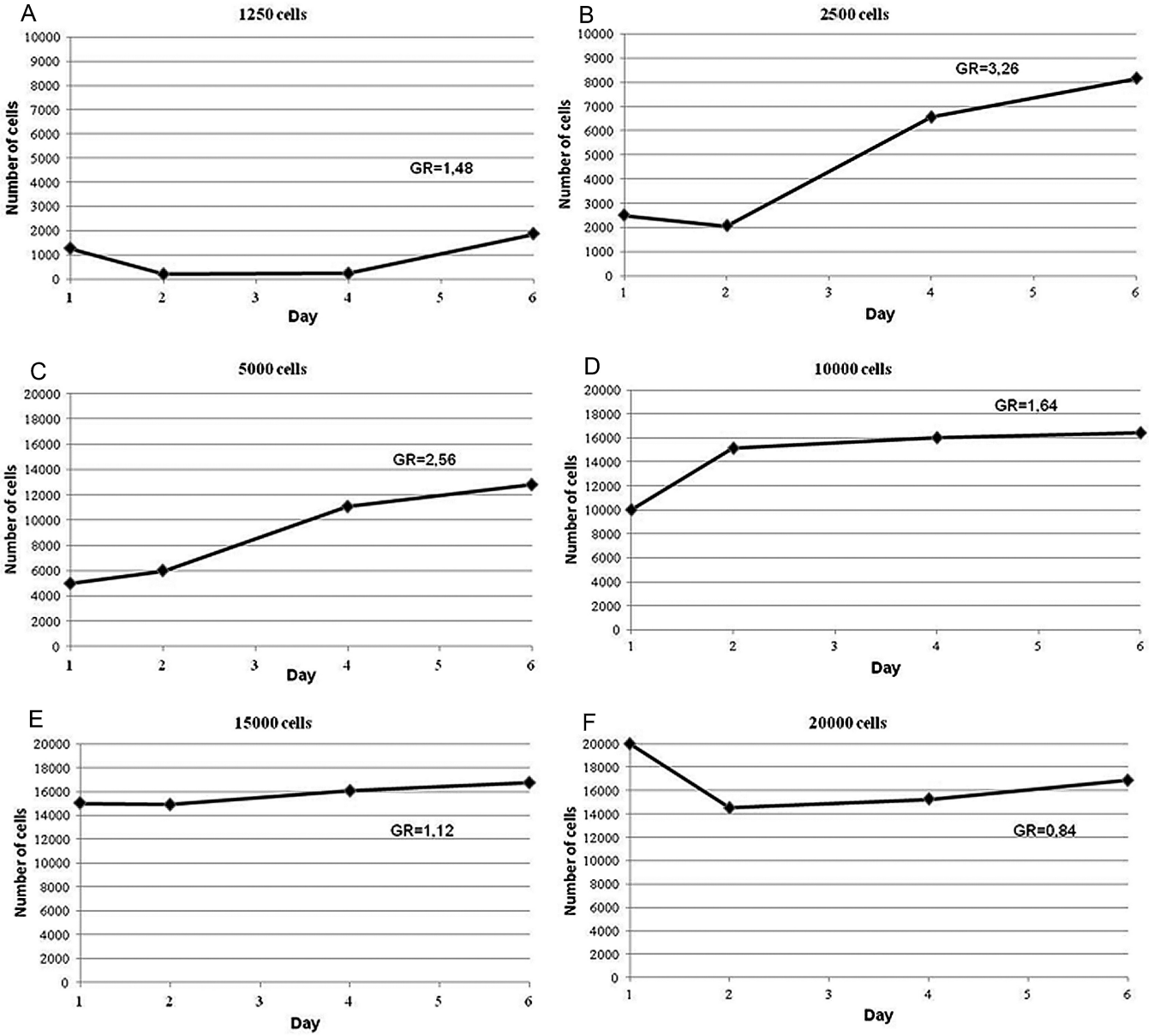

Fig. 1. Growth rate of the CMT-U27 cell line in different initial cell concentrations (range of 1250 to 20000 cells/well).

entire period of the viability assay) was a period of slow exponential growth when the cells are probably adapting to the culture environments and preparing for fast growth.

Regarding the viability assay, 10^{-2} M, 10^{-3} M and 10^{-4} M concentrations of both RU486 and ZK299 were shown to be toxic for CMT-U27 cells. Both RU486 ($p<0.05$) and ZK299 ($p<0.05$) reduced the number of viable cells at 24h at the 10^{-6} M concentration (Fig. 2). Thus, absorbance values for RU486-treated cells, ZK299-treated cells and control cells were 0.146±0.00, 0.143±0.01and 0.186±0.03 respectively. In addition, while ZK299 also reduced the number of cells (without statistical significance) at 48h, RU486 treatment increased the number of viable cells when compared to the control, also with no statistical significance (Fig. 2).

PR expression

Immunoreactive cells exhibited a nuclear staining pattern (Fig. 3). No significant differences were observed regarding intensity of PR staining between treatment groups. Twenty five percent of CMT-U27 control cells expressed PR. This figure was similar in all groups of RU486-treated cells but ZK299 treatment significantly reduced PR expression in tumour cells at 24 h of incubation ($p<0.05$) (Fig. 4). Thus, PR expression at 24 h of ZK299 treatment was just 13%. On the other hand, no differences were observed at 48h, simply a trend to increase PR expression by RU486 which did not reach statistical significance.

Discussion

The present results show that treatment of CMT-U27 cells with 10^{-6} M RU486 or 10^{-6} M ZK299 reduced the number of tumour cells. Results also show that the number of PR-positive cells decreased after ZK299 treatment exclusively. These findings show that this CMT-U27 cell line is sensitive to the effects of antiprogestins and may be useful to analyze the role of

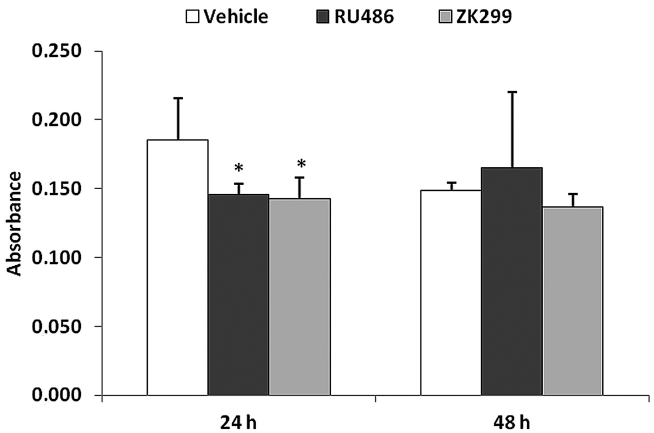

Fig. 2. Cell proliferation assay. The effect of mifepristone (RU486) and onapristone (ZK299) treatment on the viability of CMT-U27 cells at 24 and 48 hours of incubation. The results are expressed as mean ± SD. *p<0.05. Mann-Withney test.

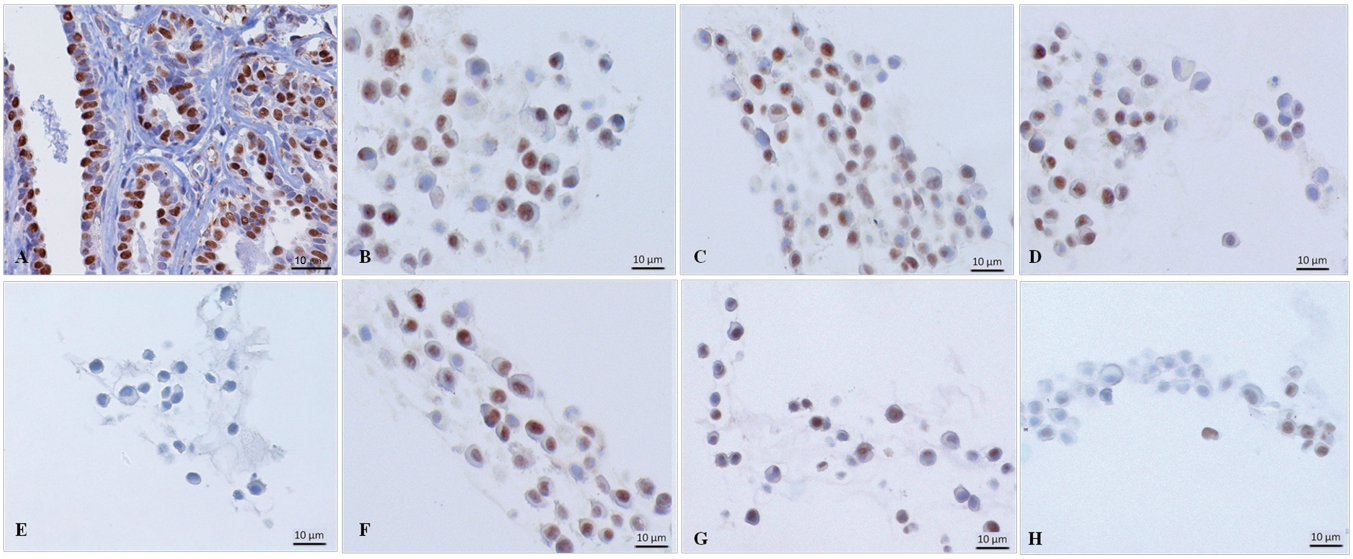

Fig. 3. PR expression by immunocytochemical assay. **A.** Canine mammary tissue (positive control with positive nuclei in brown). **B.** CMT-U27 cells treated for 24 hours with 0.01% ethanol. **C** RU486 or **D** ZK299. **E.** CMT-U27 cells, negative control (without primary antibody with negative nuclei in blue). **F.** CMT-U27 cells treated for 48 h with 0.01% ethanol. **G** RU486 or **H** ZK299. ABC method and counterstained nuclei with haematoxylin. Bar: 10 μm.

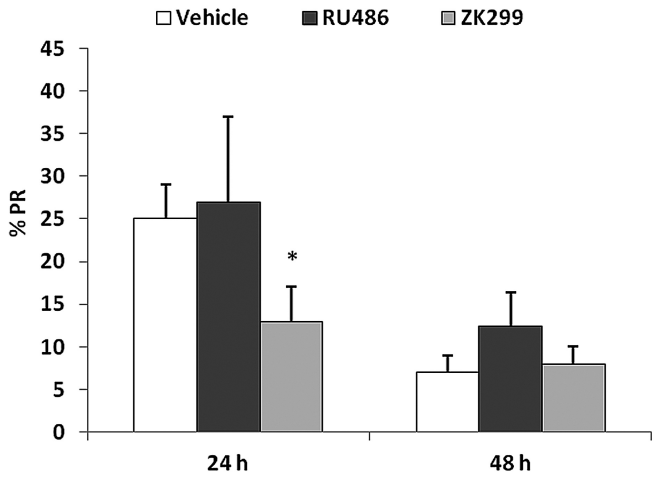

Fig. 4. PR expression related to cell treatment. Percentage of total PR positive cells in CMT-U27 cells treated with mifepristone (RU486) and onapristone (ZK299) at 24 and 48 hours of incubation. The results are expressed as mean ± SD. *p<0.05. Mann-Withney test.

these drugs in canine mammary tumours.

Accumulative evidence indicates that PR expression is a favourable prognostic indicator (Sorenmo et al., 2013; Martín de las Mulas et al., 2005) and the use of antiprogestins may be an useful tool in the treatment of canine mammary tumours (Guil-Luna et al., 2011). However, ethical and temporal requirements of *in vivo* studies make it necessary to test this hypothesis *in vitro*. The present work represents the first to address the effects of two of the most well known antiprogestins in a canine mammary carcinoma cell line.

In previous studies, the CMT-U27 cell line has demonstrated a high growth rate and anti-apoptotic potential supposed to be related to the up-regulation of several genes (Król et al., 2009). In this study, the proliferation assay with a density of 2500 cells/well showed a characteristic growth pattern of cultured cells. The first phase of growth, first 48h, is characterized by a period of slow growth and after that cells show fast growth until they reach a stationary phase at day 6.

This cell line was isolated from a *simple epithelial* carcinoma, the histological subtype with the shortest disease-free period after surgery (Misdorp et al., 1999; Martín de las Mulas et al., 2005). For this reason, *simple epithelial* carcinomas may be suitable targets for endocrine therapy in female dogs. *In vivo*, these tumours are known to have a low expression level of PR as in the present case, where some 25% of the CMT-U27 cells were labelled with the PR10A5 anti-PR antibody (Geraldes et al., 2000; Martín de las Mulas et al., 2005; Chang et al., 2009). In spite of this fact, PR-positive *simple epithelial* carcinomas not only responded to the antiproliferative effect of aglepristone *in vivo* but showed a decrease in proliferation index (Ki67-index) higher than complex and mixed carcinomas (Guil-Luna et al., 2011).

RU486 and ZK299 are antiprogestins that compete with P hormone binding at the receptor level and have demonstrated to have powerful effects on growth inhibition of mammary tumors and their metastasis in rodents and human breast cancer cell lines (Horwitz, 1992; Tieszen et al., 2011).

RU486 is a type II, partial PR antagonist that promotes PR binding to DNA (Lanari et al., 2012). In CMT-U27 cells, RU486 reduced the number of viable cells at 24 h of incubation. This finding is consistent with previous reports demonstrating a cytotoxic effect of RU486 in PR-positive human breast cancer cell lines T47D and MCF-7 (Bardon et al., 1987; Gaddy et al., 2004; Tieszen et al., 2011). Moreover, its analogue aglepristone has demonstrated to have antiproliferative effects on PR-positive canine mammary carcinomas in a *in vivo* setting. However, no *in vitro* data about this analogue of RU486 are available since it is an oil injectable solution not suitable for *in vitro* studies. It remains unclear whether the mechanism through which RU486 induces cytotoxicity in tumour cells is mediated by PR or not. In this study, we observed that RU486 had no effect on PR expression in accordance with previous findings in human breast cancer cells (Hurd et al., 1999; Navo et al., 2008). Explanations for this finding are speculative and include 1) the failure of RU486 to induce the structural changes in the receptors that lead to their down regulation by degradation through an ubiquitin-proteasome pathway once the antagonist-PR complex is created (Hurd et al., 1999; Zhang et al., 2007) and 2) the PR-independent cytotoxic activity of RU486 (Liang et al., 2003; Tieszen et al., 2011). The latter explanation is very interesting because the capacity of RU486 to decrease cell viability coupled with its lack of effect on PR expression suggests that sequential hormone therapy could be administered without any loss of efficacy. It has been demonstrated that the presence of RU486 reduced the expression of PR in human MCF-7cells and had a growth inhibitory effect maintained long after those receptors were downregulated (Tieszen et al., 2011). This latter finding discourages the role of PR as mediator of the effects of RU486. Additionally, a trend to increase the number of CMT-U27 cells was also observed at 48h for RU486 treatment. In spite of the expected antiproliferative actions of this antiprogestin on breast cancer cell lines, several reports have found that the same concentration of this antiprogestin may also have proliferative effects (Jeng et al., 1993). As this matter remains unclear, further studies in the downstream signalling pathway of this antiprogestin may help to clarify the mechanism of its effects.

ZK299 is a type I, full PR antagonist, unable to induce PR binding to P response elements or promote DNA-dependent phosphorylation. Although it has been attributed a 10-fold lower affinity of ZK299 for PR than RU486 (Hurd et al., 1999), our present results show that a concentration of 10^{-6} M ZK299 has substantial effects on CMT-U27 cells, whereas concentrations higher than

10^{-6} M were shown to be toxic for these cells, as occurred with RU486 also. Thus, 10^{-6} M ZK299 decreased both cell viability and the number of viable PR-positive cells. Numerous reports have found ZK299 to reduce cell proliferation in PR positive breast cancer cell lines (e.g. Classen et al., 1993; Iwasaki et al., 1999) and to induce quantitative alterations of PR in breast cancer cells (Hurd et al., 1999) which suggests that down regulation of PR might play a role in the effects of this PR antagonist (Hurd et al., 1999). The possibility exists that complete occupation of available PR by "pure" progestin/antiprogestins is necessary for PR down regulation. Again, the antagonist-PR complex may have induced structural changes in the receptors that lead to their degradation through an ubiquitin-proteasome pathway (Hurd et al., 1999; Zhang et al., 2007). Our present results suggest that both RU486 and ZK299 induce a decrease in the number of viable CMT-U27 tumour cells with a different effect on PR expression. However, further studies should provide evidence whether these antiprogestins suppress cell proliferation or induce cell death. Different laboratories have reported both cytostatic and apoptotic effects for both drugs in human breast cancer cell lines (Lanari et al., 2012). Finally, no significant changes were observed at 48h of treatment for any of the antiprogestins, most probably due to a degradation of these drugs in the medium. The fact that just 25% of the untreated CMT-U27 cells expressed PR might reflect some underlying heterogeneity in this cell population and clonal expansion is likely present *in vitro*. For further experimental studies on antiprogestins, the number of studied cell lines should be expanded and preferably also include cells that lack PR (Hellmén et al., 2000).

In conclusion, the canine mammary carcinoma cell line CMT-U27 is sensitive to the effects of antiprogestins and may serve to further explore the role of these drugs in canine mammary carcinomas.

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