

Aglepristone Decreases Proliferation in Progesterone Receptor-Positive Canine Mammary Carcinomas

S. Guil-Luna*, R. Sánchez-Céspedes*, Y. Millán, F.J. De Andrés, E. Rollón, V. Domingo, F. Guscetti, and J. Martín de las Mulas

Background: Progesterone receptor (PR) antagonist aglepristone (RU534) has been used successfully for pregnancy termination and therapy of pyometra, vaginal tumors, and mammary hyperplasia in bitches and queens. All of these conditions share with canine mammary carcinomas the expression of PR.

Objectives: To study the effect of RU534 on proliferation and apoptosis in canine mammary carcinomas in relation to PR expression.

Animals: Twenty-seven nonspayed bitches with mammary carcinomas were treated with either 2 doses of 20 mg/kg RU534 (n = 22, RU534-treated group) or oil placebo (n = 5, control group) on days 1 and 8.

Methods: Tumor samples were collected before (day 1) and after (day 15) treatment for immunohistochemistry. PR expression, proliferation index (PI), and apoptotic index (AI) were determined using antibodies against PR, Ki67, and cleaved lamin A/C antigens, respectively. The effect of treatment on these parameters was analyzed.

Results: Differential expression of PR between day 1 (59.1% PR-positive tumors) and day 15 (36.4% PR-positive tumors) was observed in RU534-treated tumors exclusively. After RU534 treatment, mean PI was significantly decreased in PR-positive but unchanged in PR-negative RU534-treated tumors. A reduction of $\geq 20\%$ in PI was found in 61.5% of RU534-treated tumors with PR expression. Conversely, no effect on AI was observed after RU534 treatment.

Conclusions and Clinical Importance: Neoadjuvant RU534 treatment had PR expression-related inhibiting effects on proliferation of canine mammary carcinoma cells.

Key words: Apoptosis; Dog; Progesterone receptor; Proliferation.

Surgical excision is the first-line treatment of canine mammary tumors. Approximately one-third of these tumors will recur and metastasize. However, the only type of adjuvant treatment used is chemotherapy.¹ Endocrine therapy using hormone receptor antagonists is well established for the treatment of hormone-dependent human breast cancer because it decreases relapse and metastasis rates and prolongs survival. This therapy is mostly focused on the blockade of estrogen receptors (ER) because they are expressed in approximately 70% of cases.² However, progesterone receptor (PR) expression-based therapy currently is under study for selected subsets of patients.³ Studies in dogs have demonstrated

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Abbreviations:

AI	apoptotic index
ER	estrogen receptor
H&E	hematoxylin and eosin
PBS	phosphate buffered saline
PI	proliferation index
PR	progesterone receptor
RU534	aglepristone
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

that all benign and two-thirds of malignant mammary tumors express PR.⁴⁻⁶ This finding raises the possibility of using hormone therapy with PR antagonists in these tumors.

The PR antagonist aglepristone (RU534) has been used successfully in veterinary medicine for pregnancy termination and pyometra treatment as well as to treat proliferative progesterone-dependent diseases such as mammary fibroadenomatous hyperplasia in queens and vaginal tumors in bitches. In both conditions, a tissue reductive effect has been shown.⁷⁻¹⁰ The aim of the present study was to evaluate the effect of RU534 on proliferation, apoptosis, and PR expression by immunohistochemical assessment in serial biopsies from primary canine mammary carcinomas collected before and at 15 days after the start of treatment.

Materials and Methods

Animals

A series of 27 female dogs with a histological diagnosis of mammary carcinoma were selected for this study. Additional recruited

animals with other tumor types were excluded from the present investigation. Recruitment criteria were as follows: (1) dogs were nonspayed; (2) dogs were in any phase of the estrus cycle except for estrus (as determined by vaginal cytology); (3) there were no clinical signs of inflammatory mammary carcinoma; and (4) dogs had 1 measurable mammary nodular lesion (≥ 5 mm) and had no lung metastases (as determined by 2 thoracic radiographs). All the owners gave informed consent to include their pets in this study.

Treatment Protocol

The RU534-treated group of dogs ($n = 22$) received 2 subcutaneous injections of 20 mg/kg of RU534^a on days 1 (1st visit) and 8, whereas control dogs ($n = 5$) received oil vehicle injections at the same time points. A core biopsy was taken on day 1 before to the first injection of RU534 or oil vehicle. All patients underwent complete surgical excision of the tumor at day 15.

Tissue Samples Fixation and Processing

Both core biopsies and surgical specimens were fixed in 10% buffered formalin for 24–72 hours and embedded in paraffin wax. Small tumors (< 1 cm in diameter) were entirely included, whereas sequential segments 5 mm apart were cut from larger tumors to provide tissue blocks. The area from which the core biopsy had been taken was identified by the surgeon in the surgical specimen with suture material. After dehydration and embedding in paraffin wax, sections (3 μ m) were cut from each block and stained with hematoxylin and eosin (H&E).

Histological Examination

The tumors were classified as carcinomas using H&E-stained tissue sections and the diagnostic criteria proposed by the World Health Organization Classification of Tumors in Domestic Animals.¹¹ Cases with ischemic necrosis in the incision biopsy were excluded from the present investigation.

Immunohistochemical Techniques

The monoclonal mouse anti-human Ki67 antigen (clone MIB-1) isotype IgG₁^b diluted 1 : 75, the monoclonal mouse PR antibody (clone 10A9) isotype IgG₂^c diluted 1 : 500, raised against the recombinant hormone-binding domain of human PR (922AGMVKPLLFHKK933, the sequence is 100% homologous to the canine counterpart), and the polyclonal rabbit anti-human cleaved lamin A/C (small subunit) antibody^d diluted 1 : 100 were used for the detection of proliferative activity, PR expression, and apoptosis, respectively. A commercial diluent^b was used. Heat-induced antigen retrieval in a water bath at 95–99°C (MIB-1 and PR antibodies) or in a steam pressure cooker^e at 95°C (lamin A/C antibody) with 0.01 M citrate buffer (pH 6.0) for 40 minutes (MIB-1 antibody), 25 minutes (PR antibody), or 20 minutes (lamin A/C antibody) were used. After cooling for approximately 30 minutes at room temperature, sections were covered with 10% normal goat serum in phosphate buffered saline (PBS) for 30 minutes before incubation with the primary antibodies for 18 hours at 4°C (MIB-1 and PR antibodies) or directly incubated for 1 hour at room temperature (lamin A/C antibody). The avidin-biotin-peroxidase complex (MIB-1 and PR antibodies)^f or the streptavidin biotin-peroxidase complex (lamin A/C antibody)^b were applied for 1 hour at room temperature. The chromogen, 3,3-diaminobenzidine tetrahydrochloride^g diluted 0.035% in 0.05 M Tris containing 0.3% of hydrogen peroxide was applied to the slides for 1 minute at 20–22°C (MIB-1 and PR antibody). The 3-amino-9-ethyl carbazole substrate chromogen^b was applied for 10 minutes (lamin A/C antibody). For negative control purposes, the primary antibodies were replaced by mouse IgG₁^b and IgG₂^b diluted as the primaries

(MIB-1 and PR antibodies, respectively) or PBS (lamin A/C antibody). As positive control tissues, canine lymph node, uterus, and formalin-fixed and paraffin wax-embedded ultraviolet-irradiated canine keratinocytes¹² were used for MIB-1, PR, and lamin A/C antibodies, respectively. The normal mammary gland tissue found in the vicinity of the carcinomas served as an internal positive control in every assay.

Scoring Methods

PR Expression. The staining was nuclear and the tumors were classified as positive when labeling was observed in more than 10% of tumor cells counted in 4 representative randomly selected neighboring, nonoverlapping high-power fields (approximately 1,000 tumor cells).¹³

Proliferation Index (PI). To determine the PI, digital pictures of sections labeled with the anti-Ki67 antibody were taken at a 40 \times magnification from 4 randomly selected neighboring, nonoverlapping fields of each tumor. Labeled tumor cell nuclei were considered positive regardless of the labeling intensity. The number of positive and negative tumor cells was counted with a digital pen tablet.^h A minimum of 1,000 tumor cells were counted per case. The PI was calculated with the Image-Pro Plus 4.5 softwareⁱ and expressed as the percentage of positive cells related to the total number of cells. The proliferation response of tumors to treatment was assessed in 2 different ways: (1) by comparing mean PI scores at individual time points (day 1, day 15) and (2) by classifying a $\geq 20\%$ change in PI between day 1 and day 15.¹⁴

Apoptotic Index (AI). To evaluate apoptosis, cleaved lamin A-positive cells within tumor tissue areas were counted using snapshots of 10 randomly selected fields of each tumor collected at a 40 \times magnification (Imagescope version 10.2.1.2314).^j The counts were performed independently by 2 observers and the results were averaged. The AI was expressed as the percentage of labeled cells related to the total number of tumor cells. To assess the effect of treatment on tumor cell apoptosis, results were evaluated in 2 ways analogously as described above for the PI.

Statistical Analysis

Statistical analysis was carried out by the GraphPad Software 3.05.^k The values were evaluated for approximate normality of distribution by the Kolmogorov-Smirnov test. Differences between PR expression, PI, and AI of control and RU534-treated tumors before and after treatment were assessed by unpaired *t*-tests. Differences between the means of RU534-treated tumors before and after treatment were assessed by paired *t*-tests. Correlation analyses were performed by Spearman's nonparametric correlation coefficient. A *P*-value $< .05$ was regarded as statistically significant.

Results

The animals ranged in age from 5 to 16 years of age (mean, 10.6 \pm 0.5 years) and were of various pure ($n = 14$) and mixed ($n = 13$) breeds. Histological classification of lesions is shown in Table 1. Before treatment (day 1), 60% control and 59.1% RU534-treated tumors expressed PR. A representative PR labeling of tumor cell nuclei is shown in Figure 1. A change in PR expression after treatment was observed in the R534-treated group exclusively (Table 2). Thus, 5 of 13 PR-positive tumors at day 1 became PR-negative at day 15. Conversely, 1 of 9 PR-negative tumors at day 1 was found to be PR-positive at day 15. Altogether, after treatment only 36.4% of RU534-treated tumors expressed PR.

Tumor cell proliferation and apoptosis were assessed using antibodies against Ki67 and cleaved lamin A, respectively. A representative Ki67 labeling of tumor cell

Table 1. Histological type of carcinomas.¹¹

Number	Histological Type
14	Complex carcinoma
6	Simple carcinoma
5	Carcinoma in benign tumor
1	Carcinosarcoma
1	Squamous cell carcinoma

nuclei is shown in Figure 2. Cleaved lamin A was detected in the cytoplasm and nucleus of cells with morphology consistent with apoptosis, as well as in the nucleus of some morphologically normal cells as shown in Figure 3. The overall median pretreatment PI was 7.99% (range, 1.5–16.4%) and the overall median pretreatment AI was 1.22% (range, 0.1–4.4%). No association was observed between these 2 variables ($r = 0.2$, $P = .2$). Similarly, no association was observed between PI and AI when tumors were grouped as PR-positive ($r = 0.4$, $P = .1$) and PR-negative ($r = -0.002$, $P = .9$).

PI and PR Expression

A significant decrease in PI after treatment with RU534 was observed in PR-positive tumors of the R534-treated group exclusively. Thus, as indicated in Table 3, the mean PI of PR-positive RU534-treated tumors was 7.7% before and 4.3% after treatment ($P = .03$). Seventy-eight percent of the R534-treated cases with decreased PI after treatment expressed PR. In the control group, only one of the PR-positive cases with PR expression had a reduction in PI at day 15. When a change $\geq 20\%$ of the PI was considered as a threshold, 8 of 13 (61.5%) R534-treated cases with PR expression had a PI reduction, whereas none of the cases with PR expression had a PI reduction in the control group ($P = .0003$). No significant PI increase was observed in the PR-negative R534-treated group after RU534 treatment.

No significant PI decrease was observed in both RU534-treated (7.2% at day 1, 6.9% at day 15) and control (11.0% at day 1, 9.3% at day 15) tumors ($P = .1$ and $.3$, respectively).

AI and PR Expression

No change in AI was observed either in PR-positive or in PR-negative tumors of the RU534-treated group.

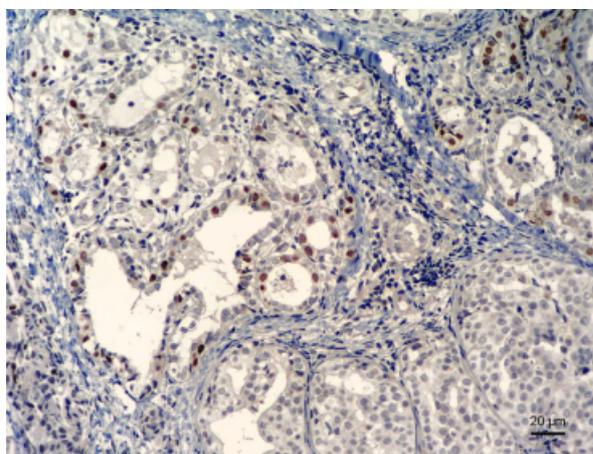


Fig 1. Simple mammary carcinoma. The nuclei of 10% tumor cells are labeled for progesterone receptors (brown color). ABC immunohistochemical method. Bar = 20 μ m.

Thus, as indicated as Table 4, the mean AI of PR-positive RU534-treated tumors was 1.5% before and 1.0% after treatment and 1.4% and 1.1 in the control group, respectively (Table 4).

Discussion

The PR antagonist RU534 decreased the PI of canine mammary carcinomas in a PR expression-dependent manner. This finding points to an influence of PR on the growth control of these tumors and raises the possibility of use of PR antagonists in the treatment of tumors of the mammary gland of the bitch.

The clinical benefits of endocrine therapy for women with hormone-sensitive breast cancer (with ER expression, PR expression, or both) are well established. Accordingly, ER and PR are measured routinely in order to select those patients who will enter the endocrine therapy protocol.² In veterinary medicine, however, no endocrine therapy, neither adjuvant nor neoadjuvant, currently is used. Former trials using tamoxifen were not successful because of strong estrogenic effects on the uterus.¹ However, the potential benefits of using PR antagonists in dogs with mammary tumors have not been explored despite the fact that PR is more often expressed than ER in the bitch.^{4–6} Ours is the first study to examine the effect of neoadjuvant RU534 in canine mammary carcinomas.

Table 2. PR expression in tumors before (day 1) and after treatment (day 15) with RU534 or vehicle (control group).

	Number of Tumors with Indicated PR Status			
	PR+		PR–	
	Day 1	Day 15	Day 1	Day 15
Control group (n = 5)	3	3	2	2
RU534-treated group (n = 22)	13	8	9	14

PR+, progesterone receptor-positive; PR–, progesterone receptor-negative.

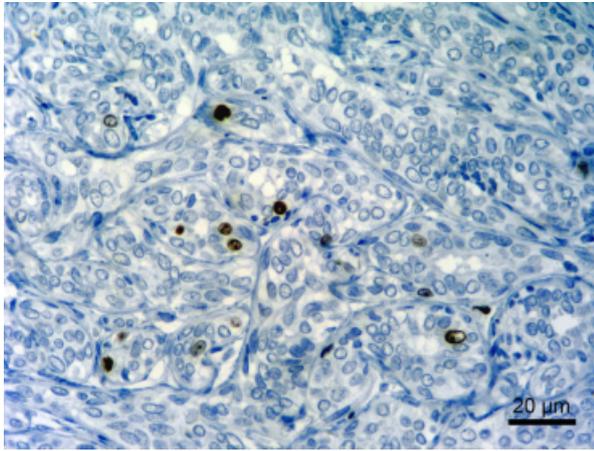


Fig 2. Simple mammary carcinoma. The nuclei of 5% tumor cells are labeled for Ki67 antigen. ABC immunohistochemical method. Bar = 20 μ m.

The proliferative response to aglepristone was related to PR status of mammary carcinomas. Thus, a significant decrease of PI on day 15 was observed in treated tumors expressing PR on day 1. Furthermore, the reduction in PI was $\geq 20\%$ in 61.5% of those cases. These results show that RU534 has an antiproliferative effect in canine mammary carcinomas with PR expression, although sample size is small to make a strong conclusion. Similar findings on PI have been reported after 14 days of neoadjuvant endocrine therapy in human patients.¹⁵ The response after only 2 weeks of treatment suggests that RU534 could be useful for the neoadjuvant treatment of canine mammary tumors.

On the contrary, treatment with the PR antagonist RU534 was not associated with a significant decrease of AI of PR-positive carcinomas. Data from RU534-treated tumors suggest that tumor growth reflects changes in the balance between apoptosis and proliferation. The *in vivo* relationship of apoptosis to proliferation is less well understood. In our material, we have

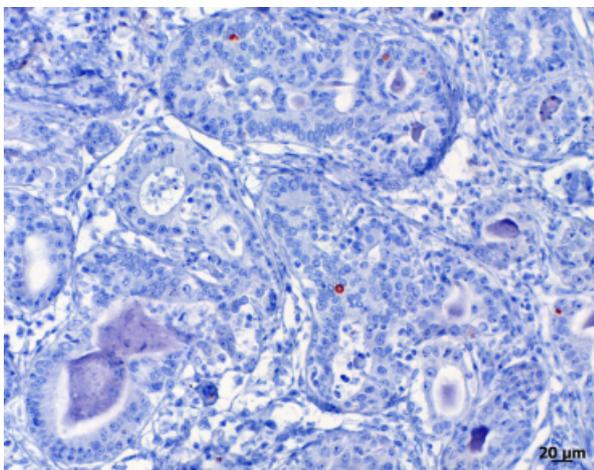


Fig 3. Complex mammary carcinoma. Single cells (1%) within the tumor are labeled for cleaved Lamin A. LSAB immunohistochemical method. Bar = 20 μ m.

Table 3. Proliferation index (PI) before (day 1) and after treatment (day 15) with RU534 or vehicle (control group) in tumors grouped according to progesterone receptor status at day 1.

	PR Status at Day 1	Mean \pm SD of PI (%)	
		Day 1	Day 15
Control group	PR+ (n = 3)	8.1 \pm 5.3	8.4 \pm 2.4
	PR- (n = 2)	15.3 \pm 1.5	10.7 \pm 2.0
RU534-treated group	PR+ (n = 13)	7.7 \pm 3.9	4.3 \pm 4.5 ^a
	PR- (n = 9)	6.6 \pm 4.7	10.5 \pm 9.0

PR+, progesterone receptor-positive; PR-, progesterone receptor-negative; SD, standard deviation.

^a $P < .05$ versus day 1. Paired *t* test.

not found any relationship between PI and AI before treatment. Other authors found a positive relationship between PI and AI of mammary gland tumors of dogs.¹⁶ However, these authors used the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method to detect apoptosis and their case material included a considerable number of benign tumors. We examined apoptosis in canine mammary tumors with a caspase substrate, a method that might be more specific than TUNEL. Lamin A is a nuclear membrane structural component of differentiated eukaryotic cells. This intermediate-sized filament protein is cleaved by the executor caspase-6 and the cleavage and subcellular localization kinetics have been described in cells undergoing apoptosis.¹⁷ In agreement with that study, immunohistochemistry for cleaved lamin A in our case material detected cells at different stages of apoptosis, with staining restricted to the nuclear membrane in putative early stages, and more dispersed nuclear and cytoplasmic staining in cells showing morphological features compatible with more advanced stages of apoptosis. In women with breast cancer, AI has been evaluated after neoadjuvant chemotherapy with inconsistent results.^{14,18} Several factors may account for these discrepancies, including the type of marker used, the type of tumors examined, the time point of tissue collection during treatment, and others.

A change in PR expression after treatment with RU534 was observed in the RU534-treated group but not in the control group. Thus, 38.5% of PR-positive tumors treated

Table 4. Apoptotic index (AI) before (day 1) and after treatment (day 15) with RU534 or vehicle in tumors grouped according to progesterone receptor status at day 1.

	PR Status at Day 1	Mean \pm SD of AI (%)	
		Day 1	Day 15
Control group	PR+ (n = 3)	2.1 \pm 1.9	2.3 \pm 1.2
	PR- (n = 2)	0.4 \pm 0.3	0.6 \pm 0.2
RU534-treated group	PR+ (n = 13)	1.5 \pm 1.3	1.0 \pm 0.5
	PR- (n = 9)	1.4 \pm 0.8	1.1 \pm 0.9

PR+, progesterone receptor-positive; PR-, progesterone receptor-negative; SD, standard deviation.

$P < .05$ versus day 1. Paired *t* test.

with RU534 became negative. These data suggest that treatment with RU534 may be associated with down-regulation of PR, a finding already described in mammary tumors of rats after treatment with the aglepristone analog RU486.¹⁹ The loss of PR may be associated with its phosphorylation after ligand binding. RU486 has been shown to phosphorylate the PR without transcriptional effects. However, phosphorylated PR is targeted for degradation.²⁰ These cells with degraded PR may have been induced to differentiate terminally.²¹ The decrease of PI after RU534 treatment of dogs bearing mammary carcinomas is compatible with the selective preservation of clones of slowly proliferating cells during treatment, as previously suggested by others studying human tumors.¹⁴

The approach of using core biopsies to assess molecular markers before and after treatment as we have used in the present study has become widely used in a number of studies in human medicine. It has, however, some limitations, including the effects of intratumoral heterogeneity in the evaluation of the response to treatment. We have tried to avoid this limitation by identifying the area where the core biopsy had been taken with suture material in the surgical specimen. In this way, tissue samples used for comparison studies were taken from the same area of the lesion. In addition, incision biopsies with necrotic areas were not used in this study. Also, the influence of overall tumor size heterogeneity on these results should be evaluated further.

In conclusion, immunohistochemical staining for Ki67, cleaved lamin A, and PR antigen has provided objective measures of the major biological effects of therapy with RU534 in canine mammary carcinoma, but further studies with both more samples and correlation data between these biological effects and clinical variables should be carried out. The present data indicate that selection for neoadjuvant endocrine therapy should be based primarily on hormone receptors status, because dogs treated with neoadjuvant endocrine therapy are most likely to respond and derive clinical benefit when their tumors are a PR-positive.

Footnotes

^a Alizine, Virbac, France

^b Dako, Barcelona, Spain

^c Immunotech, Marseille, France

^d Cell Signaling Technology, Danvers, MA

^e Pascal, Dako

^f Vector Laboratories; Burlingame, CA

^g Sigma, Saint Louis, MO

^h Volito 2, Wacom Europe GmbH, Krefeld, Germany

ⁱ Media Cybernetics, Silver Spring, MD

^j Perio, Vista, CA

^k GraphPad Software Inc, San Diego, CA

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2012	5,192	2.064	1.716	2.417	0.316	171	6.5	8.4	0.00...	0.549	94.15	Not ...	91.259
2011	4,693	1.992	1.715	2.335	0.237	190	6.1	8.3	0.01...	0.585	97.89	Not ...	90.000
2010	4,419	2.277	1.915	2.352	0.338	198	6.0	8.4	0.00...	0.500	97.47	Not ...	92.759
2009	3,887	2.168	1.800	2.262	0.204	181	5.8	8.6	0.00...	0.513	97.79	Not ...	94.718
2008	3,343	1.885	1.530	2.067	0.273	198	5.9	8.5	0.00...	0.474	97.98	Not ...	90.741
2007	3,093	1.776	1.446	2.096	0.207	188	6.1	8.8	0.00...	0.555	98.40	Not ...	91.353

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2011	15/145	Q1	90.000
2010	11/145	Q1	92.759
2009	8/142	Q1	94.718
2008	13/135	Q1	90.741