



Phase I/II Clinical Trial of Encapsulated, Cytochrome P450 Expressing Cells as Local Activators of Cyclophosphamide to Treat Spontaneous Canine Tumours

Monika Michałowska¹, Stanislaw Winiarczyk¹, Łukasz Adaszek^{1*}, Wojciech Łopuszyński², Zbigniew Grądzki¹, Brian Salmons³, Walter H. Günzburg⁴

1 Department of Epizootiology and Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences, Lublin, Poland, **2** Department of Pathological Anatomy, Faculty of Veterinary Medicine, University of Life Sciences Lublin, Lublin, Poland, **3** Austrianova, Centros, Singapore, **4** Department of Pathobiology, Institute of Virology, University of Veterinary Medicine, Vienna, Austria

Abstract

Based upon promising preclinical studies, a clinical trial was performed in which encapsulated cells overexpressing cytochrome P450 enzyme isoform 2B1 were implanted around malignant mammary tumours arising spontaneously in dogs. The dogs were then given cyclophosphamide, one of the standard chemotherapeutic agents used for the treatment of mammary tumours. The dogs were assessed for a number of clinical parameters as well as for reduction in tumour size. The treatment was well tolerated with no evidence of adverse reactions or side effects being associated with the administration of the encapsulated cells. Reductions in tumour size of more than 50% were observed for 6 out of the 11 tumours analysed while 5 tumours showing minor responses, i.e. stable disease. In contrast, the tumours that received cyclophosphamide alone showed only stable disease. Taken together, this data suggests that encapsulated cytochrome P450 expressing cells combined with chemotherapy may be useful in the local treatment of a number of dog mammary tumours and support the performance of further clinical studies to evaluate this new treatment.

Citation: Michałowska M, Winiarczyk S, Adaszek Ł, Łopuszyński W, Grądzki Z, et al. (2014) Phase I/II Clinical Trial of Encapsulated, Cytochrome P450 Expressing Cells as Local Activators of Cyclophosphamide to Treat Spontaneous Canine Tumours. *PLoS ONE* 9(7): e102061. doi:10.1371/journal.pone.0102061

Editor: Marta M. Alonso, University Hospital of Navarra, Spain

Received: May 5, 2014; **Accepted:** June 9, 2014; **Published:** July 16, 2014

Copyright: © 2014 Michałowska et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The authors have no funding or support to report.

Competing Interests: Dr Brian Salmons and Prof. Walter H. Günzburg are co-founders and employees of Austrianova. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors. All other authors report no competing interest exists.

* Email: ukaszek0@wp.pl

Introduction

The development and pathological features of spontaneously occurring mammary tumours in dogs are remarkably similar to breast cancer in women [1–3]. Indeed, the same cytological criteria that are applied in human pathology can be applied for the diagnosis of canine mammary gland tumours [4]. The incidence of these neoplasms in dogs is relatively high [5,6], and, according to recent estimates, it is the most frequently diagnosed neoplasm in female dogs, accounting for 70% of all cancer cases [7]. Mammary tumours in dogs, like those in humans, are initially hormone-dependent and malignancy correlates with a loss of hormone responsiveness [8,9]. Usually, primary mammary tumours are surgically resected. It has been reported that 58% of dogs with mammary cancer develop a new tumour after the surgical removal of the first tumour [10].

Cyclophosphamide is a standard chemotherapeutic agent that is commonly used to treat breast cancer in women [11] and has been used for this purpose also in dogs [2,12]. Successful treatment is limited by relatively low concentrations of active anti-tumour metabolites reaching the tumour, due to the necessity for activation of the prodrug cyclophosphamide by cytochrome

P450 enzymes expressed in the liver, followed by distribution of the active drug via the blood system [13,14].

We have developed a system for the local conversion of chemotherapeutic agents such as cyclophosphamide (and the related agent ifosfamide) to their toxic metabolites, rather than the usual conversion that occurs primarily in the liver [14–16]. This system consists of cells that have been genetically modified to over express the cytochrome P450 gene. The cells are both immunoprotected and localised by encapsulation in polymers of cellulose sulphate [17,18]. The cells containing the capsules are implanted either directly into the tumour or in the immediate area around the tumour. After implantation of the encapsulated cells, ifosfamide or cyclophosphamide is applied systemically. In this way elevated levels of the activated toxic prodrug metabolites are produced directly at the tumour site [19].

The DNA of all cells including the cells in the capsule are alkylated by the activated toxic prodrug metabolite phosphoramidate mustard, but the cells are only killed when they try to divide. The cells in the capsule, and also non-tumour cells, are not dividing so they are not killed. We have shown that forced growth arrest of non-encapsulated cells indeed protects them from a self-killing effect in vitro [20]. We have also characterised the mechanism of cell killing [21].

Table 1. Number of implanted CapCell and dosage of cyclophosphamide in the study group.

Female dog	Weight (kg)	Number of CapCell (day 0)	Cyclophosphamide (mg/dog)			
			day 2	day 9	day 22	day 29
VII	28	90	196	195	195	195
VIII	21	200	147	147	147	147
IX	9	750	50	50	50	50
X	12	140	87	87.5	84.7	79.8
XI	19.5	120	137	139	139	138
XII	12	200 and 320	84	84	84	77
XIII	11	94	77	77	84	84
XIV	30	450	210	210	210	210
XV	22	40	154	154	154	175
XVI	5.5	90	39	38	38	38

doi:10.1371/journal.pone.0102061.t001

Promising data have been obtained from this treatment protocol in both animal models of pancreatic adenocarcinoma [15] and in a human phase I/II clinical trial where 4 out of 14 patients with pancreatic cancer showed reductions in tumour size while the other 10 patients showed stable disease as well as a considerable survival benefit [16,22]. Encouraging data using a similar strategy has also been obtained in two immunocompetent mouse models of mammary cancer [14,23]. Based upon this data, a clinical protocol was developed for the treatment of spontaneously occurring malignant mammary tumours in dogs [24].

The current paper for the first time presents the primary data from the clinical protocol that was previously published [24]. The aim of the study was to investigate the effect of injecting cytochrome P450 overexpressing encapsulated cells around mammary tumours in dogs, followed by parenteral chemotherapy, on the cytorreduction of malignant tumour before surgery.

Materials and Methods

1.1. Dogs and tumours

A total of 16 female dogs were enrolled in the study. The dogs were divided into two groups: a study group and a control group. The control group included 6 female dogs marked as: I, II, III, IV, V, and VI. The study group included 10 female dogs marked as: VII, VIII, IX, X, XI, XII, XIII, XIV, XV, and XVI. The mean

age of the dogs that took part in the clinical trial was 8.8 years (range: 5–14 years) and the median age was 9 years. Due to the different breed of dogs included in the study, the body weight varied in the range: 9–43 kg with a mean of 18.8 kg and median of 17.8 kg. None of the 16 dogs included in the trial were spayed. Although most of the dogs (n = 13) had only a single detectable mammary tumour, four animals (IV, IX, XII, and XIII) had two tumours. Three of these dogs (IX, XII and XIII) were in the study group; whereas the dog marked IV was included in the control group.

The suitability of the animals for testing was based on the following criteria: 1) a cytological diagnosis confirming malignancy, 2) mammary tumour sizes being not less than 1 cm³ and not more than 250 cm³, 3) absence of regional metastatic lymphadenopathy, 4) absence of radiographic and ultrasonographic signs compatible with metastatic spread. The studies were reviewed and approved by the Ethics Committee of the University of Agriculture in Lublin (Poland) (Permission Number 53/2004).

1.2. Cells, capsule production and administration

A cell clone of the human embryonic kidney cell line, 293, transfected with a cytochrome P450 2B1 expression construct was used for encapsulation (CapCell) as previously described and characterised [16,20,25]. Expression of cytochrome P450 2B1 enables cells to convert chemotherapeutic agents like cyclophos-

Table 2. Dosage of cyclophosphamide in the control group.

Female dog	Weight (kg)	Cyclophosphamide (mg/dog)			
		day 2	day 9	day 22	day 29
I	20	140	140	140	140
II	40	280	280	280	280
III	40	280	280	280	280
IV	16	112	112	105	105
V	43	301	301	294	301
VI	11	77	77	84	84
VII	18	126	126	126	126

doi:10.1371/journal.pone.0102061.t002

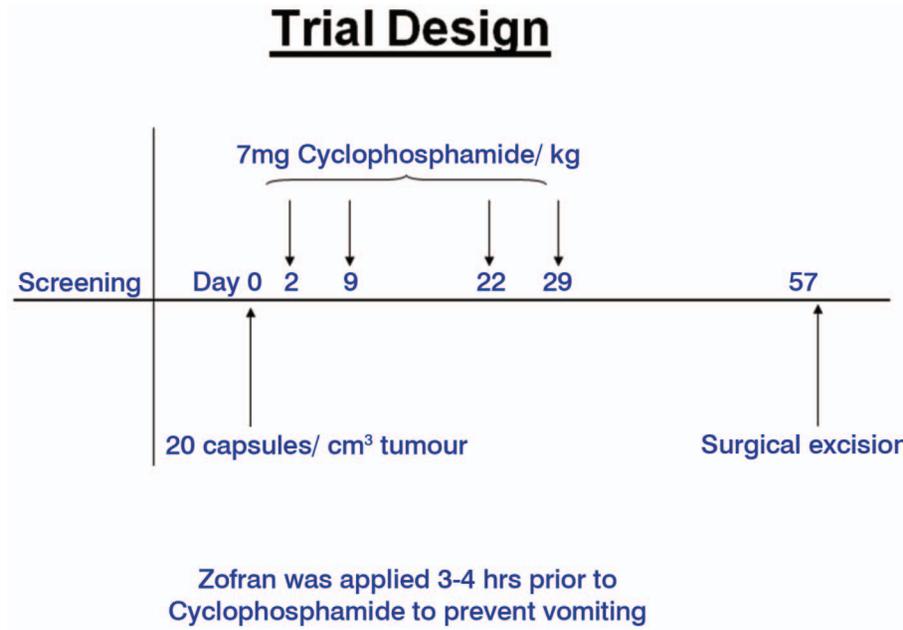


Figure 1. Design of clinical trial.
doi:10.1371/journal.pone.0102061.g001

phamide and ifosfamide to the tumour toxic metabolite phosphoramide mustard which causes DNA cross-linking. If a cell with damaged DNA tries to divide it is killed and this is the basis for the tumour selectivity of these chemotherapeutic agents [13,15].

Encapsulation of the cells was achieved as previously described [17,18] under “Good Manufacturing Practice (GMP)” conditions. Each dog received 20 cell-filled capsules per cm³ tumour, and the encapsulated cells were injected at five sites around the tumour using a G17 butterfly needle and 2 ml syringe [24]. Injections of the encapsulated cells typically required 5–10 minutes.

Although the protocol stipulated that the encapsulated cells should be applied to only one tumour in those animals with two or more tumours, the owner of one dog (XII) only agreed to the treatment if both tumours received the encapsulated cells. The other dogs with more than one tumour and not belonging to the

control group (IX, XIII) were only treated with encapsulated cells at one tumour site, in line with the clinical trial protocol [24].

1.3. Chemotherapy

The dosage and administration schedule for cyclophosphamide was based on the scheme proposed by Löscher and co-workers [26]. Cyclophosphamide was obtained as a powder (200 mg per vial, Asta Medica) and solubilised in physiological saline at a concentration of 13.3 mg/ml just prior to infusion. A total of 4 infusions of cyclophosphamide, each at a concentration of 7 mg/kg body weight, were given by intravenous infusion. The first infusion was given on day 2 after instillation of the capsules in order to allow for an observation period in case there was an acute reaction to the encapsulated cells, and the subsequent 3 infusions were given on days 9, 22 and 29 following capsule administration

Table 3. Dynamics of tumour size changes in the study group.

Female dog	Location of tumour	Tumour size (cm ³)				
		Initial	Visit 3	Visit 4	Visit 5	Visit 6
VII	R5	4.5	3.9	3.8	3.8	4.2
VIII	R3	1.0	1.0	0.9	0.7	0.7
IX	R3	37.1	34.7	28.9	28.9	28.9
X	R2	6.9	4.5	3.5	3	4
XI	L5	6.1	7.9	6.1	4.6	3.9
XII	L3	10	7.5	6.1	4.9	3.8
	L6	15.8	12.2	9.8	7.2	5.6
XIII	R4	4.7	2.7	2.5	2.5	1.4
XIV	R3	45	34.5	30.6	25.4	16.5
XV	L3	2.3	2.3	1.6	1.2	1.2
XVI	L5	4.5	2.8	1.2	1	1.5

doi:10.1371/journal.pone.0102061.t003

Table 4. Therapeutic efficiency of general administration of cyclophosphamide in the control group.

Female dog	Location of tumour	Tumour size. cm ³				
		Initial	Visit 3	Visit 4	Visit 5	Visit 6
I	L2	6.3	6.3	6	5	7.5
II	L4	244.8	242.2	221.3	203.4	174.2
III	R4	1.4	1.3	1.2	1.2	1.1
IV	L5	20.6	20.6	18.6	17.9	17.3
V	L5	4.5	4.3	3.6	3.6	3.6
VI	L4	15	15	15	13.8	13.2
XIII	R5	196	196	197	198	168

doi:10.1371/journal.pone.0102061.t004

(Tables 1 and 2, Fig. 1). Dog IX only received 79% of the dose of cyclophosphamide (50 mg) because it was 14 years old and the owner denied permission for full chemotherapy. The anti-nausea agent Zofran (GlaxoWellcome) was obtained as a ready-to-use solution and was given 3–4 hours before administration of cyclophosphamide at a concentration of 4 mg/dog (Fig. 1).

1.4. Tumour measurement

Measurements of tumour dimensions were performed with a ruler and the largest three dimensional extensions of the tumour were used to calculate the tumour volume in cm³. Measurements were taken before treatment on the day of implanting encapsulated cells (baseline) and on days 9, 22, 29 and 57.

1.5. Criteria for the assessment of treatment efficacy and safety of the treatment

The general and preliminary criteria used to assess the safety of the treatment were haematological investigation, blood chemistry and urinalysis. The haematological investigation included determination of haemoglobin level, leucocyte and trombocyte count. Blood chemistry comprised determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT) activity and blood urea nitrogen (BUN), creatinine, bilirubin and total protein

levels. Urine was analysed by means of strips and, if necessary, confirmed by routine methods.

The final result of the treatment efficacy was evaluated based on the dynamics of tumour size change according to one of the following categories: 1. Complete remission (CR), 2. Partial remission (PR) – regression of the cancer by at least 50% of the initial tumour mass, 3. Stabilization of the disease (SD) – regression of the tumour by less than 50% or its enlargement by less than 25% of the initial tumour mass, 4. Progression of the disease (PD) – increase of the tumour size by at least 25% or new neoplastic foci.

1.6. Biopsies and histology

Biopsies, using a 0.9 mm diameter needle with a 20 ml syringe, were obtained from several sites from all tumours (in dogs from the study group, as well as control group) and transferred to slides. The cell smear was immediately fixed with Fixocyt (Polish Chemical Reagents, Gliwice) and stained with haematoxylin and eosin (H&E) and Papanicolaou stain. Resected tumour samples (on 57 days of the trial) destined for histopathology were fixed in 10% neutral buffered formalin, followed by embedding in paraffin. Sections 5 µm thick were prepared and stained with H&E. Tumours were classified after routine histopathological examination according to the WHO classification system [27].

Table 5. Dynamics of tumour size change in the study group expressed as a percentage of initial tumour size.

Female dog	Location of tumour	Tumour size as % of initial size			
		Visit 3	Visit 4	Visit 5	Visit 6
VII	R5	86.7	84.4	84.4	93.3
VIII	R3	100	90	70	70
IX	R3	93.5	77.8	77.8	77.8
X	R2	65.2	50.7	43.5	43.5
XI	L5	24.6	100	75.4	64
XII	L3	75	61	49	38
	L6	77.2	62	45.6	35.4
XIII	R4	57.4	53.2	53.2	29.8
XIV	R3	76.7	68	56.4	36.7
XV	L3	100	69.6	52.2	52.2
XVI	L5	62.2	26.7	22.2	33.3

doi:10.1371/journal.pone.0102061.t005

Table 6. Dynamics of tumour size change in the control group (K) expressed as a percentage of initial tumour size.

Female dog	Location of tumour	Tumour size as % of initial size			
		Visit 3	Visit 4	Visit 5	Visit 6
I	L2	100	95.2	79.4	119
II	L4	98.9	90	83.1	71.2
III	R4	92.9	85.7	85.7	78.6
IV	L5	95.6	80	80	80
V	L5	100	90.3	86.9	84
VI	L4	100	100	92	88
XIII	R5	100	100	100	85.7

doi:10.1371/journal.pone.0102061.t006

Results

10 dogs (13 tumours) formed the study group and were treated with both capsules and cyclophosphamide.

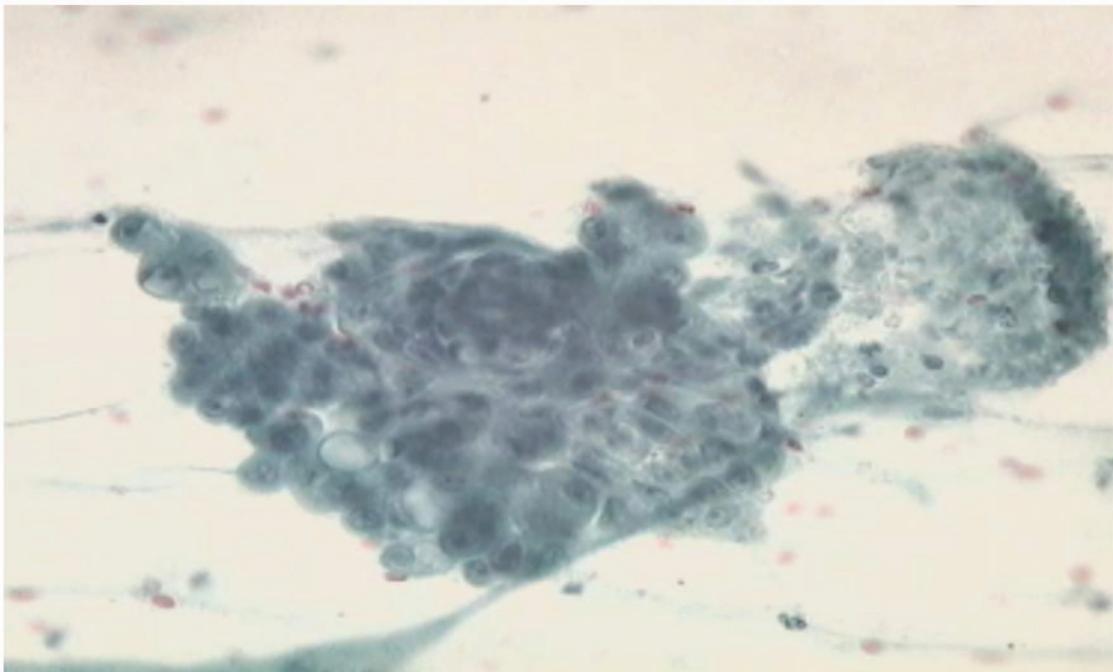
6 dogs formed the control group and were treated only with cyclophosphamide. The control group included additionally tumours in the XIII, and IX dogs without the CapCell implantation, considered an internal control site for the other tumour in these females; these tumours were taken into consideration in assessing treatment efficiency.

1.7. Safety of the treatment

Each dog in the study group received injections of the encapsulated cells around the tumour. The peri-tumour injections and the administration of the capsules were well tolerated, with no study medication related serious adverse events being observed in any of the dogs treated with the encapsulated cells over the entire observation period (56 days).

After administration of the encapsulated cells, the dogs received cyclophosphamide at a dose of 7 mg/kg. Some of the dogs (VII, IX, XI, XV) showed reduced appetite for 2–3 days after infusion of cyclophosphamide, a common side-effect of treatment with this chemotherapeutic agent [28]. The results of haematological examination of all dogs revealed mild thrombocytopenia ($163\text{--}197 \times 10^9/\text{L}$ [reference ranges $200\text{--}500 \times 10^9/\text{L}$]). In the following female dogs: V, VI, IX, XI, XII, and XVI a lower platelet count was found during the 2nd and 3rd visit, and in female dogs: III, VIII, XIII, XIV and XV during the 2nd, 3rd and 4th visit. In 5 female dogs (I, II, IV, VII, and X) thrombocytopenia persisted throughout the therapy.

Some animals (XII, II, IV, IX and XV) showed urine abnormalities such as the presence of erythrocytes and/or traces of protein. Serum creatinine and BUN levels were slightly elevated in one dog, XV (creatinine $190.0 \mu\text{mol/l}$ -reference ranges $88,4\text{--}150,3 \mu\text{mol/l}$, BUN $16.3 \mu\text{mol/l}$ -reference ranges $2,5\text{--}9,6 \mu\text{mol/l}$, and the BUN level was increased for IX ($18.8 \mu\text{Mol/L}$). AST

**Figure 2.** Results of cytology in female dog X, Papanicolaou staining, mag, x 400.

doi:10.1371/journal.pone.0102061.g002

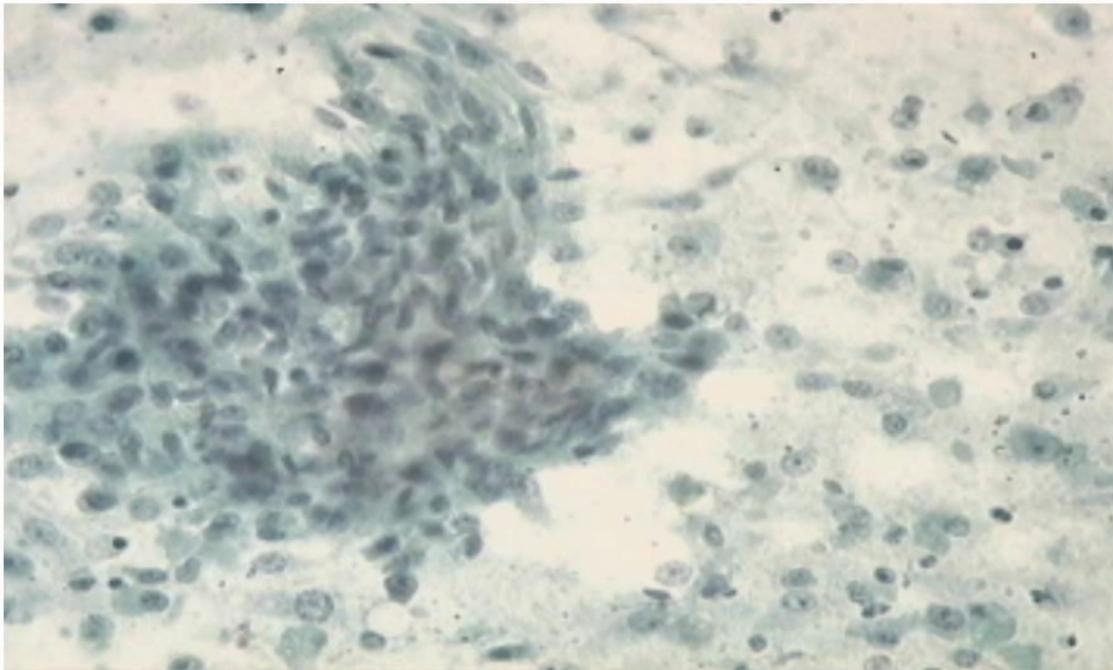


Figure 3. Results of cytology in XIII female dog Papanicolaou staining, mag, x 400.
doi:10.1371/journal.pone.0102061.g003

levels rose in 6 to 10 hours and remained above the physiological range (1–37 U) for about 4 days in all dogs of the study group. The activity of ALT was also slightly elevated in most animals (reference ranges 4–91 U), although in all cases the abnormalities for all of these laboratory parameters subsided within a few days after treatment, and were self resolving.

Thus, in summary, the application of encapsulated cells in these 10 dogs (13 tumours) was well tolerated with no obvious side-effects. The subsequent treatment with cyclophosphamide resulted in mild side effects including thrombocytopenia which has been described previously as a side effect of cyclophosphamide treatment in dogs [29,30], although these side effects were not exacerbated by the additional application of encapsulated cells.

1.8. Evaluation of tumour response/clinical efficacy

An evaluation of the tumour response was made for all of the 16 dogs (10 study group and 6 control group) participating in this clinical trial. The baseline tumour measurement (i.e. before treatment) ranged from 1.0 cm³ to 244.8 cm³ (Tables 3 and 4) with a median size of 6.6 cm³. Each tumour size at baseline was set as 100% and the size of the tumour after treatment given as a percentage of the baseline value. A total of 6 of the 11 evaluable

tumours treated with encapsulated cells and cyclophosphamide showed a partial response, as characterised by a tumour regression of about 50%. The other 5 tumours in dogs given encapsulated cells and cyclophosphamide showed stable disease (i.e. a reduction in tumour size of less than 50% or no further tumour growth) (Table 5). This data contrasts with that obtained from tumours of dogs receiving only cyclophosphamide, where 6 tumours examined showed stable disease and 1 tumour showed progression of the disease (Table 6). The largest reduction in tumour size occurred during the 4 week treatment period for both groups, although clearly treatment with the encapsulated cells and cyclophosphamide resulted in a more pronounced tumour reduction, with a median reduction of 51.3% (6,7–70,2%) as compared to 21% (0–28,8%) after treatment with cyclophosphamide alone.

More than one tumour was present in three dogs from the study treatment group (IX, XII, and XIII). Both tumours from dog XII were treated with encapsulated cells followed by cyclophosphamide and, interestingly, both tumours showed a similar reduction in tumour size (62 and 65%). It is also worthy of note that the encapsulated cell treated XIII tumour showed a reduction in size of 70%, whereas the second tumour in this dog, which did not

Table 7. The histotypes of the tumors from female dog used in the study.

Hystotype	Number of cases	Female dog
complex carcinoma	9	I, IX (2 tumours), X, XII (2 tumours), XIII (2 tumours), XVI
simple tubulopapillary carcinoma	4	IV (2 tumours), VII, XI,
simple tubulopapillary carcinoma, partially solid	3	V, VI, XV,
spindle cell carcinoma	3	II, III, VIII
in situ carcinoma comedo type	1	XIV

doi:10.1371/journal.pone.0102061.t007

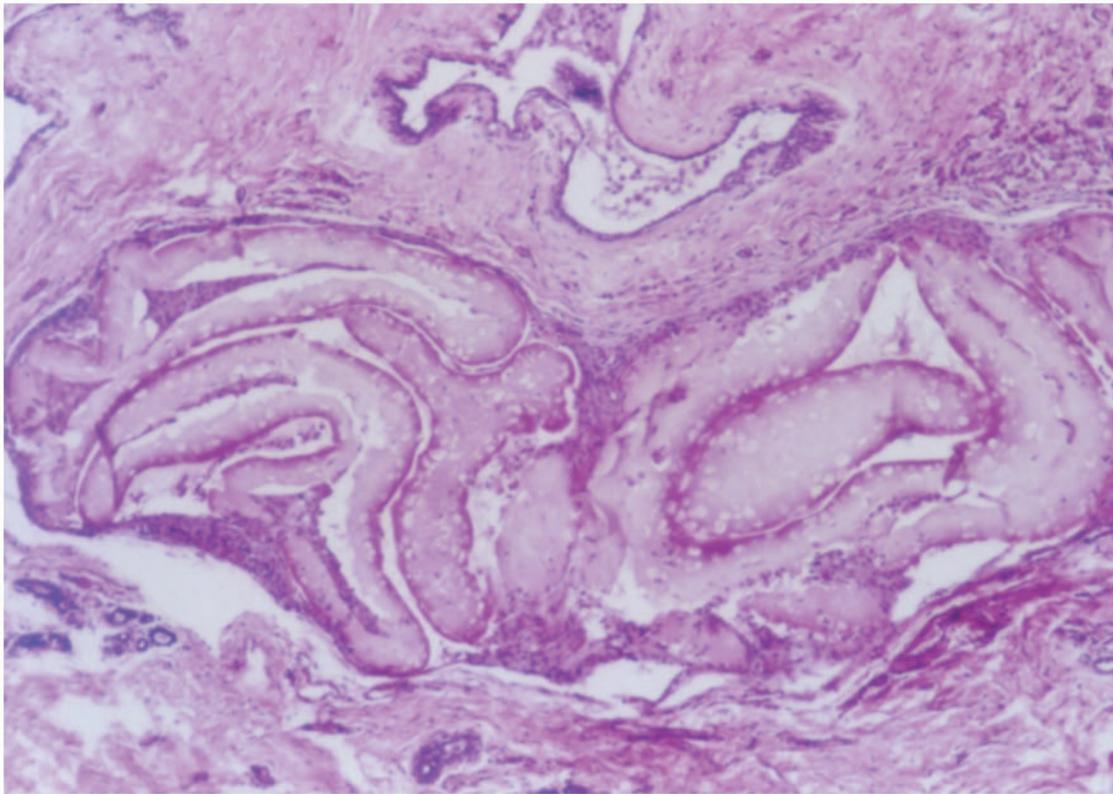


Figure 4. Section through the tumour and capsules after resection. Capsules, which have been deformed into elongated shapes by the sectioning, and the outer layer of tumour tissue are visible.
doi:10.1371/journal.pone.0102061.g004

receive encapsulated cells, only showed a 14% reduction, comparable to the reduction of 19% in tumour size in dogs from the control group.

1.9. Cytological examination

In each of the 16 female dogs that qualified for inclusion in the study, the cytological picture of biopsy specimens obtained from all the tumours indicated malignancy of the neoplastic process. At the microscopic level, the most frequently registered were the presence of necrotic masses, neutrophilic granulocytes, deformed epithelial cells and epithelial cells with a varying degree of nuclear atypia (Fig. 2, 3).

1.10. Histological analysis and resection

Twenty tumours (13 tumours from 10 dogs in the study group, and 7 tumours from 6 dogs in the control group) were histopathologically tested.

Malignant tumours were found in all dogs. In 9 cases the diagnosis was complex carcinoma, in 4 cases simple tubulopapillary carcinoma, in 3 cases simple tubulopapillary carcinoma, partially solid, in 3 cases spindle cell carcinoma, and in one case the diagnosis was in situ carcinoma of the comedo type. An overview is given in Table 7.

The encapsulated cells could be found in the tissue surrounding the resected tumours. This tissue did not show any evidence of an inflammatory reaction, although there was a slight fibrosis as demonstrated by the presence of fibroblasts and thin fibres of connective tissue. A weak infiltration of monocytes and macrophages could be observed at the border between a group of cell filled capsules and the surrounding tissue (Fig 4). Thus, these data

suggest that the encapsulated cells were well tolerated and relatively inert, thus effectively shielding the cells inside the capsule from the host's immune system. Live 293 cells could also be seen within the capsules, suggesting that the cells were non-dividing since they were not killed by the toxic metabolites of cyclophosphamide (Fig 5).

Discussion

In this study, the safety of introducing encapsulated cells capable of converting the chemotherapeutic agent, cyclophosphamide, to tumour toxic metabolites was examined. A secondary objective was to determine whether this local conversion had a better therapeutic effect than the usual cyclophosphamide treatment that relies on conversion of the chemotherapeutic agent by cytochrome P450 enzymes expressed in the liver.

Although side effects of the treatment could be observed, none of these were due to the implantation of encapsulated cells but rather to the administration of cyclophosphamide at the standard dose, since they were also experienced by the control animals receiving cyclophosphamide only. Histological sections of material resected from the tumour and surrounding areas showed that the encapsulated cells were well tolerated, with no or only mild immune reactions. These findings are in line with those from previous studies, both from a clinical phase I/II trial for pancreatic cancer in human patients [16,31,32], as well as in animal models [14,15,19,33]. In this respect, it is worth mentioning that even encapsulated hybridoma cells producing a monoclonal antibody [34,35] or encapsulated retroviral vector producing cells [36] have been shown to be well tolerated after implantation into

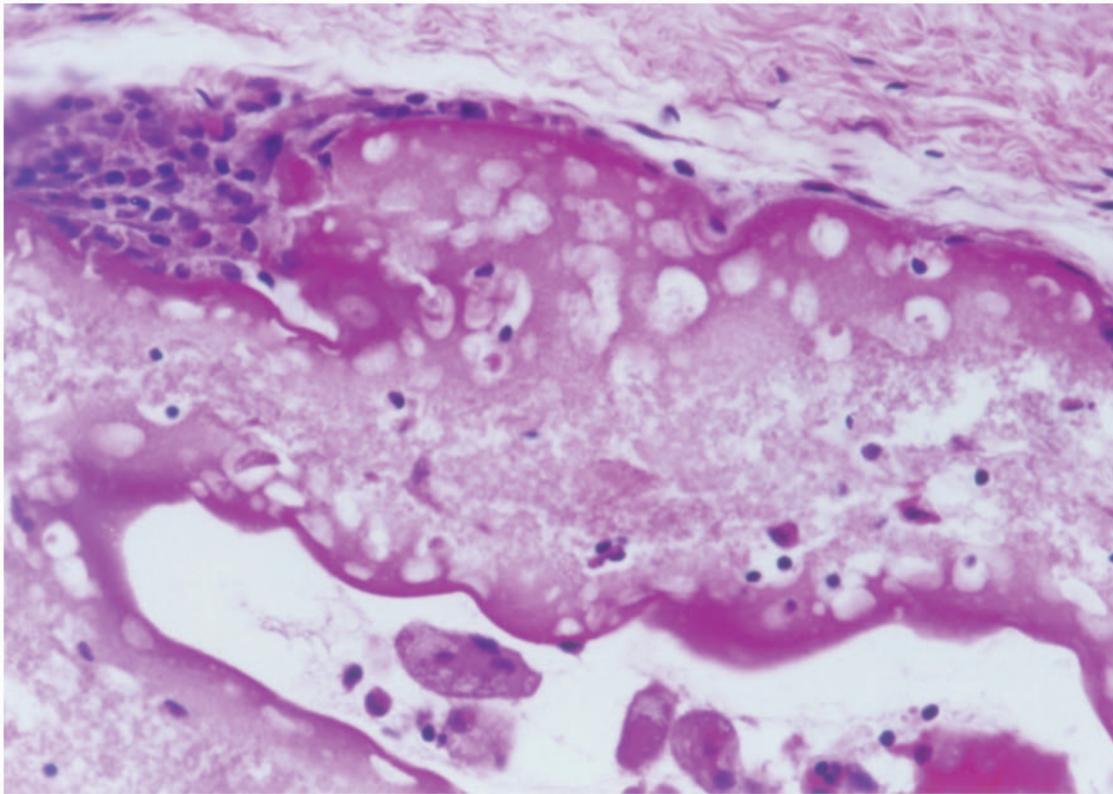


Figure 5. Close up of a capsule, revealing living HEK293 cells inside.

doi:10.1371/journal.pone.0102061.g005

immunocompetent mice. Nevertheless, immune responses can result if xenogenic cells are released, for example by capsule rupturing [37].

In studies performed in patients with inoperable pancreatic cancer, the microcapsules were angiographically placed in the blood vessels leading to the tumour. In the mammary cancer study reported here the microcapsules were injected peri-tumorally. Since similar tumour shrinkages were observed in both studies, this argues strongly that the results observed in the human clinical trial were not caused by vessels being blocked by microcapsules, but rather from the focusing of the toxic effect of cyclophosphamide on the tumour. In follow-up studies, similarly to the study reported here, no allergic reactions or signs of pancreatitis were observed in these patients. These results demonstrate that microcapsules effectively protect the encapsulated cells against attack by the host's immune system whilst allowing free diffusion of nutrients from the external environment into, and the release of cellular metabolites out of, the microcapsule. [34,35,37,38].

In this study, peri-tumoral injection of encapsulated cells, followed by systemic application of cyclophosphamide, was used in a neo-adjuvant setting to shrink tumours before they were surgically removed. This improves the success of the surgical excision since less skin has to be removed, hence improving wound closing and thus the healing process [39]. A long term benefit may be expected since a smaller tumour is generally associated with a better prognosis, even after surgical removal has taken place [2,9]. It should also be possible to combine the encapsulated cell therapy

with other treatments such as gemcitabine, which has recently been shown to give significantly improved overall survival rates, but only when given in an adjuvant setting after surgical resection [40]. Such a combination trial might be of interest since 58% of dogs with mammary cancer develop a new tumour after the surgical removal of the first tumour [10].

Conclusion

The use of encapsulated drug converting cells for improved, local chemotherapy is a safe treatment strategy. The use of this strategy results in improved anti-tumour effects as measured by tumour reductions, suggesting that it may be useful for the treatment of mammary tumours in human and animal patients. Thus, further clinical studies are warranted.

Acknowledgments

We would like to thank Barbara Kosztolich for her help in the analysis of the data and Robert M. Saller for his help with capsule production.

Author Contributions

Conceived and designed the experiments: SW BS WG. Performed the experiments: MM LA WL. Analyzed the data: SW ZG BS WG. Contributed reagents/materials/analysis tools: MM SW LA WL ZG BS WG. Contributed to the writing of the manuscript: LA WL BS SW.

References

- Owen L (1979) A comparative study of canine and human breast cancer. *Invest Cell Pathol* 2: 257–275.
- Sorenmo K (2003) Canine mammary gland tumors. *Vet Clin North Am Small Anim Pract* 33: 573–596.

3. Munson L, Moresco A (2007) Comparative pathology of mammary gland cancers in domestic and wild animals. *Breast Dis* 28: 7–21.
4. Cassali GD, Gobbi H, Malm C, Schmitt FC (2007) Evaluation of accuracy of fine needle aspiration cytology for diagnosis of canine mammary tumours: comparative features with human tumours. *Cytopathology* 18: 191–196.
5. Brodey RS, Goldschmidt MH, Roszel JR (1983) Canine mammary gland neoplasms. *J Am Anim Hosp Assoc* 19: 61–90.
6. Perez-Alenza MD, Tabanera E, Pena L (2001) Inflammatory mammary carcinoma in dogs: 33 cases (1995–1999). *J Am Vet Med Assoc* 219: 1110–1114.
7. Merlo DF, Rossi L, Pellegrino C, Ceppi M, Cardellino U, et al. (2008) Cancer incidence in pet dogs: findings of the Animal Tumor Registry of Genoa, Italy. *J Vet Intern Med* 22: 976–984.
8. Donnay I, Rauis J, Devleeschouwer N, Wouters-Ballman P, Leclercq G, et al. (1995). Comparison of estrogen and progesterone receptor expression in normal and tumor mammary tissues from dogs. *Am J Vet Res* 56: 1188–1194.
9. de Las Mulas JM, Millán Y, Dios R (2005) A prospective analysis of immunohistochemically determined estrogen receptor alpha and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog. *Vet Pathol* 42: 200–212.
10. Stratmann N, Failing K, Richter A, Wehrend A (2008) Mammary tumor recurrence in bitches after regional mastectomy. *Vet Surg* 37: 82–86.
11. Gordon A (1997) The increasing efficacy of breast cancer treatment. *Clin Oncol* 9: 338–342.
12. Karayannopoulou M, Kaldrymidou E, Constantinidis TC, Dessiris A (2001) Adjuvant post-operative chemotherapy in bitches with mammary cancer. *J Vet Med A Physiol Pathol Clin Med* 48: 85–96.
13. Chen L, Waxman DJ, Chen D, Kufe DW (1996) Sensitization of human breast cancer cells to cyclophosphamide and ifosfamide by transfer of a liver cytochrome P450 gene. *Cancer Res* 56: 1331–1340.
14. Kammertoens T, Gelbmann W, Karle P, Alton K, Saller R, et al. (2000) Combined chemotherapy of murine mammary tumors by local activation of the prodrugs ifosfamide and 5-fluorocytosine. *Cancer Gene Ther* 7: 629–636.
15. Löhr M, Müller P, Karle P, Stange J, Mitzner S, et al. (1998) Targeted chemotherapy by intratumour injection of encapsulated cells engineered to produce CYP2B1, an ifosfamide activating cytochrome P450. *Gene Ther* 5: 1070–1078.
16. Löhr M, Hoffmeyer A, Kröger J, Freund M, Hain J, et al. (2001) Microencapsulated cell-mediated treatment of inoperable pancreatic carcinoma. *Lancet* 357: 1591–1592.
17. Dautzenberg H, Schuldt U, Grasnick G, Karle P, Müller P, et al. (1999) Development of cellulose sulfate-based polyelectrolyte complex microcapsules for medical applications. *Ann N Y Acad Sci* 875: 46–63.
18. Löhr M, Hummel F, Faulmann G, Ringel J, Saller R, et al. (2002) Microencapsulated, CYP2B1-transfected cells activating ifosfamide at the site of the tumor: the magic bullets of the 21st century. *Cancer Chemother Pharmacol* 49 Suppl 1: S 21–24.
19. Salmoms B, Günzburg WH (2010) Therapeutic application of cell microencapsulation in cancer. *Adv Exp Med Biol* 670: 92–103.
20. Günzburg WH, Karle P, Renz R, Salmoms B, Renner M (1999) Characterization of a human cell clone expressing cytochrome P450 for safe use in human somatic cell therapy. *Ann N Y Acad Sci* 880: 326–336.
21. Karle P, Renner M, Salmoms B, Günzburg WH (2001) Necrotic, rather than apoptotic, cell death caused by cytochrome P450-activated ifosfamide. *Cancer Gene Ther* 8: 220–230.
22. Salmoms B, Löhr M, Günzburg WH (2003) Treatment of inoperable pancreatic carcinoma using a cell-based local chemotherapy: results of a phase I/II clinical trial. *J Gastroenterol* 15:78–84.
23. Uckert W, Salmoms B, Beltinger C, Günzburg WH, Kammertöns T (2004) Combination suicide gene therapy. *Meth Mol Med* 90: 345–352.
24. Winiarczyk S, Gradzki Z, Kosztolich B, Gabler C, König G, et al. (2002) A clinical protocol for treatment of canine mammary tumours using encapsulated, cytochrome P450 synthesizing cells activating cyclophosphamide: a phase I/II study. *J Mol Med* 80: 610–614.
25. Karle P, Müller P, Renz R, Jesnowski R, Saller R, et al. (1998) Intratumoral injection of encapsulated cells producing an oxazaphosphorine activating cytochrome P450 for targeted chemotherapy. *Adv Exp Med Biol* 451: 97–106.
26. Löscher W, Ungemach FR, Kroker R (1994) Immunosuppressiva. In: *Grundlagen der Pharmakotherapie bei Haus- und Nutzieren*. 2. Aufl., Parey verlag, Berlin, pp 398–399.
27. Misdorp W, Else RW, Hellmen E, Lipscomb TP (1999) Histological classification of the mammary tumours of the dog and the cat. In: *World Health Organization International Histological Classification of Tumours of Domestic Animals*, F.I Shulman, Ed., Armed Forces Institute of Pathology, Washington DC pp.18–29.
28. Elmslie RE, Glawe P, Dow SW (2008) Metronomic therapy with cyclophosphamide and piroxicam effectively delays tumor recurrence in dogs with incompletely resected soft tissue sarcomas. *J Vet Intern Med* 22: 1373–1379.
29. Nemoto K, Ogino M, Sugawara Y, Abe F, Ito J, et al. (1994) Improvement of mitomycin C- and cyclophosphamide-induced thrombocytopenia and leucocytopenia by prior treatment with deoxyspergualin in dogs. *Br J Haematol* 87: 572–575.
30. Zenker I, Meichner K, Steinle K, Kessler M, Hirschberger J (2010) Thirteen-week dose-intensifying simultaneous combination chemotherapy protocol for malignant lymphoma in dogs. *Vet Rec* 167: 744–748.
31. Löhr M, Klöppel G, Maisonneuve P, Lowenfels AB, Lüttges J (2005) Frequency of K-ras mutations in pancreatic intraductal neoplasias associated with pancreatic ductal adenocarcinoma and chronic pancreatitis: a meta-analysis. *Neoplasia* 7: 17–23.
32. Löhr M, Bago ZT, Bergmeister H, Cejka M, Freund M, et al. (1999) Cell therapy using microencapsulated 293 cells transfected a gene construct expressing CYP2B1, an ifosfamide converting enzyme, instilled intra-arterially in patients with advanced-stage pancreatic carcinoma. A phase I/II study. *J Mol Med* 77: 393–398.
33. Kröger JC, Benz S, Hoffmeyer A, Bago Z, Bergmeister H, et al. (2003) Intra-arterial instillation of microencapsulated, ifosfamide-activating cells in the pig pancreas for chemotherapeutic targeting. *Pancreatol* 3: 55–63.
34. Pelegrin M, Marin M, Noël D, Piechaczyk M (1998) Genetically engineered antibodies in gene transfer and gene therapy. *Hum Gene Ther* 9: 2165–2175.
35. Pelegrin M, Marin M, Oates A, Noel D, Saller R, et al. (2000) Immunotherapy of viral disease by in vivo production of therapeutic monoclonal antibodies. *Hum Gene Ther* 10: 1407–1415.
36. Saller RM, Indraccolo S, Coppola V, Esposito G, Stange J, et al. (2002) Encapsulated cells producing retroviral vectors for in vivo gene transfer. *J Gene Med* 4: 150–160.
37. Armeanu S, Haessler I, Saller R, Engelmann MG, Heinemann F, et al. (2001) In vivo perivascular implantation of encapsulated packaging cells for prolonged retroviral gene transfer. *J Microencapsul* 18: 491–506.
38. Stange J, Mitzner S, Dautzenberg H, Ramlow W, Knippel M, et al (1993) Prolonged biochemical and morphological stability of encapsulated liver cells—a new method. *Biomater Artif Cells Immobilization Biotechnol* 21: 343–352.
39. Novosad CA (2003) Principles of treatment for mammary gland tumors. *Clin Tech Small Anim Pract* 18, 107–109.
40. Marconato L, Lorenzo RM, Abramo F, Ratto A, Zini E (2008) Adjuvant gemcitabine after surgical removal of aggressive malignant mammary tumours in dogs. *Vet Comp Oncol* 6: 90–101.