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Review

New Advances on Canine Bladder Cancer: Immunotherapy and Nanotechnology Applications

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Abstract: Human urinary bladder cancer has similar development that invasive urothelial cancer that happens spontaneously in dogs. Then, research of these malignant tumors associated with the advances of new treatment drive to the specific fight of the urothelial tumor cell, is highly valuably related to efficiency and reduction of side effects of treatments, even extrapolating to humans. Thus, a review of the most important aspects on new advances on canine bladder cancer was achieved. Many forms of therapies are being reported; one of them, of highly promising feature is the treatment with immunomodulators. The resemblance between spontaneous urothelial cancer in canines and urinary bladder cancer in humans makes this canine tumor relevant animal model to research and advances of treatment that may be more efficient in management of tumor with less unfavorable effects. Immunomodulators exhibit important outcomes when used in the therapy of bladder cancer in dogs. Nanomaterials that are possible carriers of some kinds of drugs to the target tumor significantly raise the efficiently of the therapy. Therefore, the advancement of immunomodulator combined with nanoparticles can disrupt the therapy of bladder cancer in dogs and thereafter in humans.

Keywords: bladder cancer; canine; immunotherapy; nanotechnology

1. Introduction

An important review demonstrated that canines with some naturally happen cancer could be extremely significant animal models to support classic models. In the case of invasive urinary bladder cancer (IUBC) or invasive urothelial carcinoma (UC) or invasive transitional cell carcinoma (TCC) in canines appeared with similarity to cancer in humans pathology, biological behavior and molecular aspects containing some types of metastasis, and replication to chemotherapy.[1] Alongside all these parameters, the genomic tests were described with similarities among invasive UC in canines and incase of humans.[2] The appearance of mutations in several genes involved in human invasive UC have been determined also in dogs.[3] The overexpression of epidermal growth factor receptor (EGFR) family, appears as a particularly interesting target, and this parameter has been confirmed by immunohistochemistry (IHC) in 73% of canine UC cases, close to that in humans and a canine trial of an EGFR-targeted compound.[4] Resembling, expression of androgen and estrogen receptors has already been reported[5] and in *microarray studies* (GEO database), there were over >400 genes expressed between normal and tumor either in dogs and humans.[6] It is significant to point out that BC comprehend around 2% of all spontaneously cancers in canines, near identical to human´s rate.[7] There are 89.7 million pet canines in the United States and a 25% cancer evolving older dogs, and probably BC will affect more than 20,000 dogs/year.[8] The following in these cases is by histologic exam, cystoscopy, or catheter biopsy.[7] The great amount of invasive UC in canines, possibly over > 90% of the cases are of intermediate- to high-grade invasive UC.[9] The last year an overview of the UC grading systems available in both human and veterinary pathology was reported and exhibited a detailed description and a critical evaluation to help veterinary researchers in to define a grading

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system to apply in future studies on canine UCs.[10] Interesting, it is extremely uncommon in canines a superficial, low-grade invasive UC.

Along these facts, the resurgence of immunotherapies in cancer therapy emerges that canine models that also have a level of immune system like to that in human cancer individuals make this model relevant to comprehend the effect of immunomodulators in humans.[5,11,12]

Publications on immune checkpoints in canine tumors exist an indication that are crucial point in human cancer, then, there is important interest in expanding canine-specific treatment antibodies to exert a blockage of these checkpoints.[13-17] The vision that a relevant oncology research could enhance the success of human clinical trials day by day is a clear evidence of that is important and not less relevant the new advances in immunotherapies is also of great importance.[18] According to Rebhun et al.[19], recent research revealed significant findings through immunotherapy: it was tolerated well by dogs and still in a 2-week course of inhaled IL-15 resulted in held suppression of malignant and diffuse metastatic cancer. They concluded that in near future clinical application, IL-15 could be used in a multimodal strategy coupled with other therapies.

The purpose of this review is to collect the initial assays and the new immunotherapeutic therapies on canine a present in progress and the nanotechnological techniques on BC.

2. Immunomodulators or BRMs

BRMs are administrated with the aim of improve the antigenic stimulation of host immune response, either from an exogenous or endogenous source.[20] BRMs involved cytokines and extrinsic or synthetic molecules, such as constituents and mitigated live bacteria, such as *Bacillus Calmette-Guérin* (BCG).

2.1. BCG

The therapy of tumors to live attenuated strain of *Mycobacterium bovis* BCG enhances tumor cell expression of MHC (major histocompatibility complex), therefore inspiring immunological finding. Simply, stimulation of Th1 (T-helper Type 1) cell generation of cytokines and tumor necrosis factor alpha) (TNFα) improves antitumor action of natural killer (NK) cells, cytotoxic T lymphocytes and macrophages and the generation of antitumor memory T cells.[21] Almost 50 years ago, instillation of BCG application to bladder was published to diminish regression of superficial BC in humans[22] and then many publications in the 1970- 1980s evaluated BCG in tumor-bearing dogs, with variable outcomes.

A work published a case of an undefined breed bitch (10 years old), exhibiting a bladder carcinoma attended with antineoplastic chemotherapy (cisplatin), cyclooxygenase-2 (COX-2) inhibitor together with BCG demonstrated an efficiency for the non-tumor progress. Intravesical cisplatin alone, in this case no satisfactory response was observed. Due to the unavailability and difficulty of acquisition, BCG was excluded from the treatment, and the dog was maintained with carboplatin and COX-2 inhibitor. Two months after discontinuation of BCG, there was an increase in the intravesical nodule, as well as the appearance of a new mass in the ventral portion of the urinary vesicle. Then, the application of BCG demonstrated tolerable outcomes in the canine, after that ceased tumor progression and also metastasis. Seemingly emerges as a hopeful method for the regulation of neoplasms in canine.[23]

2.2. Salmonella typhimurium

A systemic application of mitigated of *Salmonella typhimurium* (forty one tumors) (spontaneous neoplasia) in canines were administrated by i.v infusion; 15% demonstrated an effective response. The unfavorable effects were not significant but got more important at higher concenrations.[24]

2.3. Cytokines

It was published that interleukin-2 (IL-2) was necessary for clonal expansion of antigen-specific naive T cells, and induces proliferation, interferon (IFN)-γ generation and cytolytic activity of NK

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cells and macrophages.[26] Thus, veterinary investigators have assumed this type of approximation targeting. Authors, such as Khanna et al.[27] applied recombinant human (rh) IL-2 encapsulated in liposomes and 2 canines showed lengthy total remission and another canine demonstrated lengthy stable illness on lung cancer. It was not observed important adverse effects.

A Intralesional interleukin-2 (IL-2) on the clinical monitoring of tumor evolution in canines experiencing from urinary bladder and urethral carcinomas was studied. From 25 canines diagnosed with advanced TCC, 14 canines, were treated by intralesional IL-2 via transabdominal ultrasound-guided application and 7 canines suffered cytoreductive surgery, followed by IL-2 injection into the tumor bed. 14 canines were administrated with non-steroidal anti-inflammatory drugs. No unfavorable response related to IL-2 applications were found and 17 canines demonstrated a significant clinical enhancement and regression of the tumor. Four canines were in total remission. Intralesional IL-2 administration was a secure and a minimum invasive palliative therapy option.[28] Rudokas et al.[29], however, pointed out that advances of anticancer liposomes for inhalation probably depend on the advanced development of effective devices in order to reach a maximum the benefit of treatment and diminish the potential for punctual site and systemic negative effect.

Several cytokines have showed potentiality *in vitro* assays and in preclinical models. Recombinant human IL-12 (Rh IL-12) enhanced proliferation of canine peripheral blood mononuclear cells (PBMCs) and increased allogeneic tumor cell lysis by PBMCs *in vitro*.[30]

Reported that there are many benefits from the application of Cell-Wall Fraction of Mycobacterium in TCC canines. The association of chemo-therapeutic-like (apoptosis) and immune-therapeutic (cytokine induction) action in Cell-Wall Fraction of Mycobacterium (CWFM) is single one. In addition to this, the chemotherapeutic-like action of CWFM arises to be self-sufficient of attendance of the multiple medicine opposition (atypical or typical) and to Fas-ligand opposition or p-21, p53, and p-16 mutations, all of that interfere with the activity of several of typical chemotherapeutic medicines. Fundamentally based on this remark observation from *in vitro* and *in vivo* reports showing biological action against canine TCC, additional forthcoming reports researching application as a possible alternative or further therapeutic option for canine TCC were guarranty.[31]

While few human cytokines are operative in canines, these efficiently are neutralized by antibodies against them.[32] In a report, the advancement of canine IL-15 has been published reacting on melanoma.[33,34] But the anti-tumor action was restricted, however, this report is an excellent case of how canines can help to delineate of studies in humans and guidance medicine progress.

2.4. Natural Killer Cells

Previously reported that human NK cells are determined by the expression of CD56 and absence CD3 and also by the T-cell receptor (TCR).[35] Meanwhile, on contrary, canine NK cells did not express those kinds of biomarkers. Natural killer cells (NKs) default the feature of T-lymphocyte markers CD3 and besides this express CD5dim.[36,37] The hourly growing of NK-92 (human NK cell line) can supply an unlimited number of ready to use NK cells and has recently been demonstrate to cross-react with canine cancer targets.[38,39] In canine cases was published that only blood lymphocytes have been used as NK cell origin. Ames et al.[40] demonstrated that NK cells extracted from blood lymphocytes administrated intra-tumor can carried to tumor return. Besides that, a spontaneous cytotoxicity (differently to T-cells, in which no priming is needed) the NK cells are relevant effectors cells in mAB (monoclonal antibodies) interjacent antibody-dependent cellular cytotoxicity, an action mode that, in general, in humans counts for almost of the anti-tumor action of mAbs.

The fermentation of a hybridized mushroom enhanced NK cell activity through an extract of active Hexose Correlated Compound (AHCC).[41] A male Labrador (12-year old) with a malignant diagnosis of BC was admistrated AHCC.[42] After progression of BC, the canine did not exhibit frequent urination and presented a good clinically monitoring initiation of AHCC therapy. Therapy also demonstrated imortant higher immune response but as a negative implication of a progressive renal failure, the levels tended to diminish. The improvement in the immune response of AHCC through NK cell activity was shown.[43]

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2.5. Immunomodulator (P-MAPA)

Recently, various experimentally induced animal models of bladder cancer have been set up, including chemically cause tumors[44,45], including P-MAPA therapy (an immunomodulator).[45-47] Spontaneously taking place BC in canines may supply an outstanding model, due to is quite closely mimics human IBC.[5] For this purpose, P-MAPA immunotherapy actions on the advances of BC were evaluated by our group in 2 canines (Fávaro et al., unpublished results). Two female Teckel breed (Dog 1 and Dog 2), with around 12 and 14 years old. They were attended at a Veterinary Clinic after urothelial carcinoma diagnosis and consent of canine owners. P-MAPA therapy was initiated after allowed by Institutional Committee for Ethics in Animal Use-CEUA-UNICAMP, protocol no. 4258-1). The canines were treated with P-MAPA in physiological saline solution by cystocentesis. Dog 1 exhibited the presence of tumor mass with irregular morphologies, hyperechoic echotexture, mixed echogenicity, covering almost the entire bladder volume (see Table 1). The tumor mass was diminished almost 91% after 6 instillations of P-MAPA related to the initial ultrasound. In Dog 2 was found tumor mass penetrating and very similar to Dog 1, with a 62% in volume after 6 instillations of P-MAPA 9Table 1). At the end of treatment, no loss of weight after P-MAPA treatment. Thereby, these outcomes indicated that P-MAPA intravesical immunotherapy was efficient in decreasing andavoiding the progression of urothelial neoplastic damages in spontaneous BC.[46-47]

Table 1. Urothelial tumor size and corporal weight of Dog 2, before and after P-MAPA instillations.

P-MAPA Treatment	Corporal Weight	Urothelial Tumor Size
Before the first instillation	6.05kg	2.69 cm x 1.28 cm
After the first instillation	6.03kg	2.21 cm x 1.39 cm
After 3 instillations	6.15kg	2.21 cm x 1.06 cm
After 6 instillations	6.1kg	1.07 cm x 0.86 cm
After 8 instillations	6.07kg	0.83 cm x 0.62 cm
After 10 instillations	6.09kg	1.1 m x 0.61 cm

2.6. Nanoimmunomodulator (Oncotherad)

In a study evaluating the efficacy and toxicological effects of OncoTherad intravesical immunotherapy in 6 canines with BC it was shown that before the initial of first instillation of OncoTherad, they exhibited an irregular tumor mass, hyperechoic echotexture, and mixed echogenicity (tumor size of 9.38 cm^3). During the 6th instillations of OncoTherad, it was found a reduction of around 62% of its volume, and at the end of 24 instillations, exhibited a reduction of around 84% of its volume. Hematuria was disappeared at 8th applications. Interesting, OncoTherad treatment did not showed any signs of systemic toxicity at the therapeutic dose. Thus, OncoTherad intravesical immunotherapy appeared safe and effective option for spontaneous canine BC and may supply benefit for avoiding tumor recurrence.[48]

2.7. Monoclonal Antibodies (mAbs)

The monoclonal antibodies (mAbs) acting on CTLA-4 (checkpoint molecules cytotoxic T-lymphocyte–associated antigen 4) and PD1 (programmed cell death-1) induce an efficiency effect in humans, among others, the bladder cancer.[49] CTLA-4 as also PD-1 are manifested into T lymphocytes and control in a negative form the immune action, that PD-1 is also manifested in canine lymphocytes[17]. PD-L1 is the link for PD-1 on tumor cells which is no expressed on normal cells. Researchers utilize canine tumor biopsy samples and resembles with human mAb attest the manifestation of PD-L1 on several canine tumors.[50] But, a relative new clinical trial with a mAb on canine PD-L1 demonstrated a very restricted activity in canines with forward melanoma.[13]

Due to the attendance of high velocities of somatic mutations exist a great resistance in therapy of invasive UC. These types of cancers is possible to avoid immune vigilance and elimination through the manifestation of programmed cell death-ligand 1 (PD-L1; or B7-H1 or CD274) around the tumor microenvironment. Anti-PD-L1 antibody atezolizumab (MPDL3280A) was assayed, an efficient

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systemic cancer immunotherapy, for the therapy of metastatic phase of urinary cancer. A high-affinity engineered human anti-PD-L1 monoclonal immunoglobulin-G1 antibody, Atezolizumab, restricts the action of PD-L1 with PD-1 (PDCD1) and B7.1 (CD80). Due to PD-L1 is manifested on activated T cells, atezolizumab was prepared by an engineering process with a transformation in the Fc domain (fragment crystallizable region) corresponds to the tail zone of an antibody that reacts with cell surface receptors (Fc receptors) and only some proteins of the complement system, which suppress antibody-dependent cellular cytotoxicity at clinically important concentrations to avoid the decreasing of T cells expressing PD-L1. Thus, it was demonstrated that atezolizumab had important action in metastatic urinary BC. Answers were frequently fast, around 6 weeks, with many happening at the time of the first replay evaluation.[51]

Unlikely, anti-PD antibodies did not exhibit efficient anti-tumor actions in various animal patterns in the initial days of the study, enhancing skepticism between many over their therapeutic therapy. The reason for this it was that the potentiality of anti-PD treatment relies on both the presence of immune cells, especially T cells in the tumor place as also of PD-L1 responce by the cell´s tumors.[52]

2.8. Viscum album (Mistletoe, Family Loranthacae)

The *Viscum album* is commercialized under mark names Iscador (or Iscar), Iscucin, Lektinol, Helixor (called Plenosol) and abnobaVISCUM. Among them, Helixor® mistletoe formulations were approved for adjunctive therapy of human cancer in Germany and in many others countries.

Helixor® has been demonstrated to exhibit immune-protective, immuno-modulating and anti-mutagenic activities. Enhances the neutrophils and the number of NK cells; it increases IFN-γ; it causes cytokine liberation (IL1, 2 and 6 and TNFα); *in vitro* is cytotoxic; exhibits DNA stabilizing action; it induces T cells and the generation of GM-CSF (granulocyte-macrophage colony-stimulating factor); it increases fibroblastic activity which restores matrix; it increases novel collagen production and limits collagenase action of cancer cells, and it defenses peripheral blood lymphocytes to immunosuppressant and chemotherapy-induced mutagenicity. All these actions justify mainly of Helixor's clinically settled effects of release of C-related protein (CRP), fever, leucocytosis and other severe phase proteins, and enhancement of medullary haematopoeisis. Inhibitions tumor growth effects outcome in a diminishing or finishing of tumor growth. Researchers have used Helixor® extracts from 1984 for cancer treatment in canines and cats. Therapy is initiated with daily subcutaneous administrations.[53]

Therapy with intravesical instillation of mistletoe extract as process in humans with non-muscle invasive bladder cancer (NMIBC) was demonstrated to be secure and extremely well tolerated.[54-56]

Diverse Viscum formulations have been assayed in *in vitro* as well as *in vivo* models, but unfortunately no further information were given on canine BC therapy[7]

3. Platelet- Rich Plasma (PRP)

Most actual approach to cancer is Immunotherapy.[58] On this direction, molecules that exert modulation on immune system, through TLRs (Toll-like receptors), appears as an important strategy for the cancer therapy, if used single or in association with known therapies.[59] TLRs exerts important functions in innate immunity that after activation can activate two different replays in tumors: first stimulation of immune system to kill tumor cells and/or delete the inhibitory apparatus to the immune system.[60,61] Regarding to the role of TLRs modulation in the tumor´s treatment comprising NMIBC, it was researched if PRP could include some activities on control of these receptors. It is known that PR, by definition, that is a platelet lysate, intended in a tiny volume of plasma, concentrating of several potential growth factors (GFs), such as: TGF-β (transforming growth factor); PDGF (platelet-derived growth factor[62]: EGF (epidermal growth factor, bFGF (basic fibroblast growth factor); VEGF (vascular endothelial growth factor; HGF (hepatocyte growth factor); IGF (insulin growth factor) and also cytokines. It is relevant in several healing/ tissue repair stages processes.[63] It was confronted the conventional therapy for cervical ectopy (laser therapy) with the treatment based on activated PRP gel in humans.[64] The outcomes demonstrated identical treatment

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efficiency for both treatments, however the side effects was significantly lower in the PRP therapy. The actual PRP mechanism in squamous reepithelialization of cervical ectopy is not actually completely understood. But, same researchers suggested that reepithelialization of cervical ectopy could be assigned to GFs appearance in the PRP (e.g., IGF-1, PDGF, TGF-β, VEGF, FGF) that function as control agents, promoting chemotaxis and proliferation and cellular differentiation.

A study reported the effects of a promissory treatment possibility for NMIBC in the presence of BCG intravesical immunotherapy associated with PRP in a murine model (rats). NMIBC was caused by treatment Fischer 344 rats (female) with N-methyl-N-nitrosourea (MNU).[46,47] After MNU´s treatment, the rats were allocated into 4 groups, such a control (no MNU) group, an induced cancer group (MNU), a cancer group in the presence of PRP, a cancer group treated with BCG and a cancer group treated simultaneously with PRP and BCG. Results showed that single PRP treatment or combined with BCG exhibited cytotoxicity in bladder carcinoma cells (HTB-9). Rats treatment with PRP combined to BCG neatly demonstrated improvement histopathological recovery from the cancer conditions and diminishes of urothelial neoplastic lesions progression in 70% of rats compared to groups which received only the same therapies administered alone. Also, this therapeutic combination drove to different activation of immune system TLRs 2 and 4-mediated, resulting in raised TRIF, IRF3, MyD88 and IFN-γ immunoreactivities. Then, with the information obtained indicate that the activation of interferon signaling pathway by PRP therapy in association with BCG immunotherapy could be a new therapeutic approximation for NMIBC in canines.[65,64] Experiments with spontaneous canine bladder cancer are under progress at Biology Institute at University of Campinas, Brazil.

4. Nanotechnology Applications in Canine Bladder Cancer

4.1. Chemotherapy

Nanotechnology was applied in order to enhance the liberation effectivity of paclitaxel in intravesical treatment to BC.[66] Lu et al.[67,68] published a paclitaxel-loaded gelatin protein nanoparticle (named Abraxane®) for intravesical treatment. In the canine treatment, they showed that an intravesical dose of Abraxane® can liberate around 3-fold higher medicine amount than Cremophor formulated paclitaxel in the urothelium surface and lamina propria tissue layers. Then, Abraxane® can give a more expanded liberation for intravesical treatment. This Abraxane® nanoparticles, in clinical trial phase I was applied in intravesical therapy for BCG unamenable/refractory NMIBC [69], and presented negligible toxicity and systemic absorption in unhealths.

The use of deracoxid in clinical trial to treat canines with TCC of the urinary bladder showed the deracoxid's efficiency and tolerability, with a survival ratio times confront to those initially published using other treatment dosages.[70]

HA-Pt (hyaluronan-cisplatin nanoconjugate (20-25 nm) preparation that liberate cisplatin to tumors after intra- and peritumoral administration was published. The efficacy and safety of HA-Pt in a small Phase I/II clinical trial acting on tumor-bearing canines was evaluated. HA-Pt reached a 23% complete effect in canines with locally progressed heterogeneous head and neck cancers. Interesting HAPt did not produced nephrotoxicity, which is the early toxicity for platinum drugs in canines. In this sense a new-generation HA-Pt with enhanced hepatic safety and similar anti-cancer efficiency is being produced.[71] These nanostructure merges as interesting in the application of canine BC.

4.2. Immunotherapy: OncoVeter (Veterinarian formulation of Oncotherad)

Interesting outcomes has been demonstrated that OncoTherad, a drug, which is a nanostructured immunomodulator acted efficiently on bladder cancer.[72-82]

In a study in which treatment of 6 female dogs diagnosed with BC showed that before the first application of OncoVeter, all patients exhibited amorphous tumor mass, hyperechoic echotexture and mixed echogenicity, with an average tumor volume of 9.38 cm^3 (by ultrasound).[83] A significant efficiency was observed after 6 instillations of OncoVeter (tumoral mass reduction over 62%). A

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reduction of 85% (1.37 cm^3 tumoral volume) was found (Table 2). A 83.33% of the patients exhibited stable disease, 16.66% partial remission. A 100% of patients at the end of treatment exhibited partial remission of the diseases (24th application). After 402-day follow-up with Oncotherad relapse-free/progression-free survival rate was 100%.

Table 2. Comparison of tumor volume throughout the therapeutic regimen (ANOVA for repeated measures with the response variable transformed into ranks). Different lowercase letters (a, b, c, d, e) indicate significant differences across treatment. Inst = instilation.

Variable	N	Average	D.P.	Mínimum	Median	Máximum	p-valor
Tumoral volume_before1Inst	6	9.38[a]	4.43	4.07	9.00	14.93	**<.0001**
Tumoral volume_after1Inst	6	6.59[b]	2.05	3.75	6.55	9.12	
Tumoral volume _ater3Inst	6	5.68[b]	2.20	3.51	5.56	9.15	
Tumoral volume _after6Inst	6	3.06[c]	1.46	1.60	2.67	5.77	
Tumoral volume _after18Inst	6	.09[d]	0.89	1.17	1.91	3.79	
Tumoral volume _after24Inst	6	1.37[e]	0.78	0.63	1.21	2.83	

Different lowercase letters (a, b, c, d) indicate significant differences across treatment. App = application. D.P. = standard deviation.

Besides of all these data OncoVeter showed no toxicity at this therapeutic dose. Then, OncoVeter immunotherapy exerts a safe and efficient therapeutic possibility for spontaneous canine BC and providing advantages for preventing tumor regression.

5. Conclusion

The likely aspects in spontaneous urothelial cancer in canines and urinary BC that affects humans suggests that this canine tumor a greatly pertinent animal model for the invention and advancement of therapies that may be highly efficient in attack tumor with less negative response. Between these therapies, immunomodulator molecules or biological response modifiers exhibit pertinent results when applied in the therapy of BC in canines. Treatments with BCG or combined with BCG, therapies with cytokines, NK cells, compounds that raise the liberation of cytokines and the action of NK cells, and therapies which modulate the immune system over TL receptors, appears promising methods for the control of various neoplasms in canines and, obviously, in humans. Moreover, the application of nanoparticles acting as carriers of some kinds of medicines to the target tumor highly raises the effectiveness of the therapy. Thereby, the advancement of biological response modifiers combined with nanoparticles can represent a disruption process in the treatment of BC in canines and consequently in humans, once the immunotherapeutic process will have an augmented absorption associated with low toxicity, alongside its enforcement being target-directed cancer.

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