Chapter 9

Air Pouch Model: An Alternative Method for Cancer Drug Discovery

Moisés Armides Franco-Molina,
Silvia Elena Santana-Krímskaya and
Cristina Rodríguez-Padilla

Additional information is available at the end of the chapter

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Abstract

The tumor microenvironment (TME) is composed of cancer, immune, and stromal cells that interact through cell-to-cell contact and a diverse milieu of cytokines, chemokines, growth factors, and proteases. Several reports have linked the presence of specific cell subtypes with tumor stages, prognosis, and patient survival. Understanding cancer cell behavior and their response to treatment within the tumor microenvironment is essential to prevent establishment, growth, and progression of tumors. Many synthetic and biological agents have been tested using cell-based assays, which do not provide reliable predictive capacity for drug candidate performance in vivo. In this chapter, we discuss about the benefits of an air pouch tumor model, in which tumor cells are inoculated inside an air pouch created on the back of the mouse. The air pouch cancer model serves as a confined/localized tumor microenvironment, where direct contact of drug candidates and tumor cells is guaranteed in a tumor microenvironment context. Therefore, the efficacy of the therapeutic agent can be accurately assessed in vivo.

Keywords: tumor, air pouch, mice, in vitro, in vivo, antitumor

1. Introduction

Cancer research requires a significant amount of in vitro and in vivo preclinical studies. In vitro cancer cell line cultures are routinely used as the first step for evaluating potential efficacy for cancer drugs and therefore determine the "stop/go" decision for drug development,

followed by animal and finally human trials [1]. However, only about 5% of anticancer drugs finally get approved for clinical use due to lack of clinical efficacy or intolerable toxicity [1, 2].

In vitro approach contribution for biotechnological development is undeniable; however, cell lines are maintained in nonphysiological conditions that do not resemble body temperature, electrolyte concentration, extracellular matrix contact, cell density, and heterogeneity. Culture conditions also implicate sudden changes such as media exchange and nutrient depletion. Furthermore, rapid cell growth is desirable and induced, driving cell subpopulations not to differentiate. All of these factors alter cell signaling and favor specific subpopulations of cells that adapt to these artificial conditions and loose some of their original characteristics [3].

To optimize cancer drug screening, it is necessary to include the biological and genetic components that influence cancer treatment outcome. Therefore, to study the cancer cell in vivo, it is essential to understand how to prevent the establishment, growth, progression, and metastasis of cancers and how to modulate the tumor microenvironment (TME) for therapeutic gain [4].

Mouse models have been used to assess toxicology and efficacy of anticancer drugs, as well as for the study of tumor induction (establishment), progression, and metastasis; these traditional mouse models have been successfully used to identify cytotoxic drugs that are still the main anticancer treatment in therapy today [4, 5].

In this chapter, we discuss about the benefits of a tumor model in which tumors can grow inside an air pouch created in the dorsal part of the mouse (**Figure 1**). The air pouch cancer model serves as the local microenvironment, which can be modulated to study establishment, progression, and metastasis as well as the efficacy of therapeutic agents.

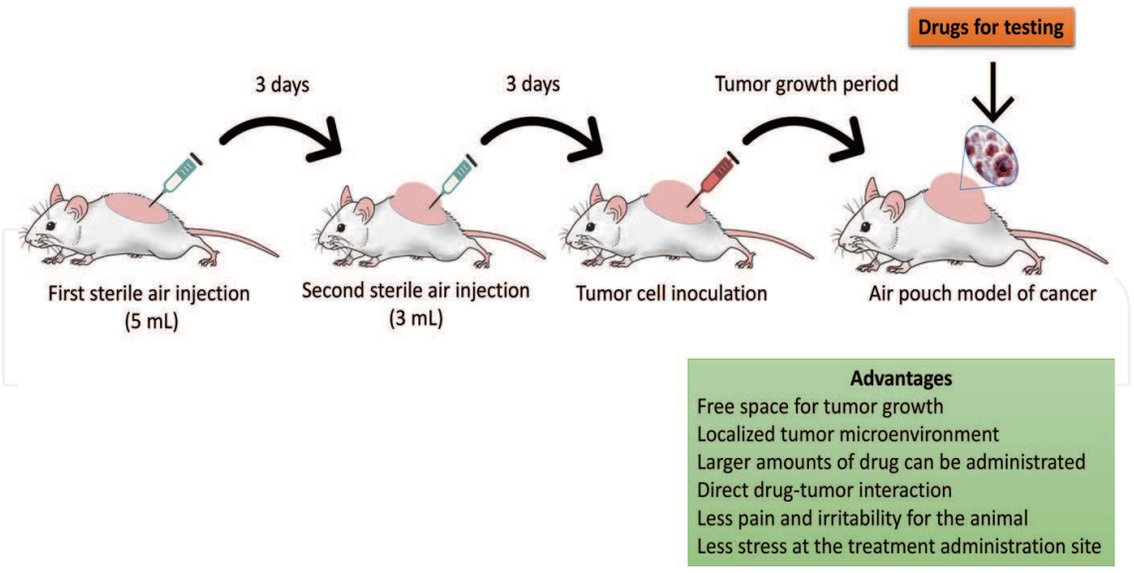

Figure 1. Mouse air pouch model for the study of cancer scheme. The general methodology to induce the air pouch model for the study of cancer and its advantages is depicted. Briefly, 5 milliliters of sterile air is injected into the shaved back of a mouse; after 3 days the pouch is reinflated with 5 milliliters of sterile air. After 3 days tumor cells are inoculated and allowed to grow prior to drug administration.

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2. Model review

1953: The air pouch model was described by Selye for the study of the mechanism of action of hydrocortisone. Selye termed his model the granuloma pouch model and described it as a "procedure designed to permit the objective, quantitative analysis of factors regulating inflammation and wound-healing" [6].

1954: Kleinfeld and Habif evaluated the anti-inflammatory effect of trypsin and chymotrypsin inside a granuloma pouch. According to the authors, this method provides a standardized and reproducible inflammatory lesion. They evaluated the volume of the exudate and the weight of the pouches. The authors concluded that parenteral administration of trypsin and chymotrypsin does not affect the formation of granulation tissue in a chronic sterile inflammation environment [7].

1956: Hewitt observed that the number of viable cancer cells needed to induce tumors in half of an experimental group of adult mice was 1641 and in newborn mice was 10. He hypothesized that tumorigenesis could be affected by the dispersion of cancer cells and by the vascularity of the injection site. To test this hypothesis, he injected tumor cells into subcutaneous tissue and into an air pouch, and to evaluate the vascularity effect, he induced hyperemia in air pouches with a formic acid solution. Hewitt concluded that neither cancer cell dispersion nor vascularity affected the capacity of cancer cells to induce a tumor. He also suggested the use of the air pouch technique for further tumor transplantation studies [8].

1957: Selye wrote another article this time addressing the question: is inflammation good or bad for cancer development? He induced two air pouches on the back of the same animal, administered croton oil into one of the pouches to establish an inflammatory environment, inoculated cancer cells into both pouches, and evaluated which tumor developed faster. The results showed that inflammation can promote tumor development [9].

1957: A scientific report stated that the air pouch model presented several advantages over other methods for the study of inflammation. According to Robert and Nezamis, the granuloma pouch technique is simple and yields uniform results, and it is quantifiable because the degree of inflammation is reported in grams of the tissue, thickness of the pouch membrane, or volume of exudate. There is no systemic response, and furthermore they recommend this technique as a screening test for unknown compounds because minimal amounts of substance can be tested with high sensitivity in a short period of time [10].

1957: Based on the observation that two concomitant tumors in the same animal inhibited or promoted their development, Hans Richer inoculated nonviable sarcoma cells and viable Walker tumor cells into a mouse air pouch, to determine if the growth inhibition between tumors was due to nutrient competition. The air pouch model was used in this study because, according to the author, it is an ideal method to test a localized effect. He concluded that nonviable sarcoma cells delayed tumor growth when injected previously or concomitantly with the viable Walker tumor [11].

1958: It was known that cortisol retarded the growth of Walker tumor in rats; to determine if the effect was due to a local or systemic action of the hormone, Selye used his air pouch technique. According to him, the "granuloma pouch technique forces the malignant tissue to grow in the form of a thin lining on the wall of a regularly shaped ellipsoid cavity to which accurately measured amounts of hormones can be applied." Selye concluded that tumor growth inhibition was considerably more pronounced when cortisol was administered inside the air pouch containing the tumor as compared to administration subcutaneously in another location. In the same article, the author suggests that the hormones probably do not act directly upon cancer cells, but rather modify the stroma and the vascularization [12].

1958: Another adaptation of the air pouch model was used by Ship et al. to determine whether administration of local cytotoxic agents could prevent tumor recurrence after surgical interventions. For a better recreation of a surgical wound environment, Ship removed the air out of the pouches after tumor inoculation. He termed his modified model "the air bubble technique" and demonstrated the effectiveness of formaldehyde against tumor cells implanted in vivo [13].

1958: The granuloma pouch was used to study the role of histamine as a mediator of acute inflammation. The air pouch model was employed because, according to previous reports, croton oil used to form the granuloma pouch disrupted mast cells, therefore releasing histamine. The histological changes associated with inflammation were not observed when histamine was depleted. The author concludes that histamine is closely related to early inflammatory reactions [14].

1961: Thoracic duct lymph cells from donor rats were injected into an air pouch of a receptor rat, for cytological and histological studies of the lymphocyte. The inoculation of thoracic lymph duct cells into the air pouch also allows simultaneous injection of antigen, fatty substances, and other materials to stimulate development of plasma cells and macrophages, among others, and either donor or pouch animal can be pretreated to alter the lymph cells or their tissue environment [15].

1967: Chang, Gibley, and Ichinoe used the air pouch to study the histological features of early passages of a chemically induced hepatocarcinoma. The authors stated that the air pouch method provides easy access to the tumor for faster processing of the tissue for histochemical, ultrastructural, or biochemical studies [16].

1967: The effect of the hormones progestogen, estrogen, and chorionic gonadotropin on the inflammatory response was studied. The granuloma pouch technique (air pouch with croton oil injection) in rats was used as the inflammation model. The thickness of the granuloma pouch was the parameter used to measure inflammation. It was concluded in this study that progestogen, estrogen, and chorionic gonadotropin act as antiphlogistic substances on acute inflammation [17].

1968: Orchidectomized adult male rats received a subcutaneous transplant of ovarian grafts. The experiment determined that daily injections of progesterone (0.1 milligrams) and continuous mild stress (air pouch with croton oil) or higher doses of progesterone (0.25 milligrams) induce luteinization [18].

1968: Another adaptation of the air pouch model was employed by Toto et al. to determine if artificially produced cell culture media could induce cell proliferation in vivo. This group administered tritiated thymidine into the mouse to evaluate the proliferation rate. Their results suggest that T199 tissue culture medium supports and promotes cell growth in vivo [19].

1969: The air pouch model was used to examine the role of polymorphonuclear leukocytes in tissue repair. Briefly, an air pouch was formed in the back of mice, and turpentine was injected to cause acute inflammation. Polymorphonuclear leukocytes were extracted from this air pouch, labeled with tritiated thymidine, and inoculated into the pouch of a recipient mouse. Leukocyte cytolysis was detected and the tritiated thymidine was incorporated by DNA synthetizing fibroblasts. The authors concluded that the neutrophil degradation products are used by fibroblast to proliferate and repair damaged tissue [20].

1969: Willis used the air pouch model to obtain large volumes of exudate to extract and quantify prostaglandins for further assays. He suggested that pharmacological activity of inflammatory exudates was attributed to E-type prostaglandins [21].

1970: Anti-inflammatory activity of topically applied hydrocortisone acetate, methylprednisolone acetate, betamethasone 17-valerate, triamcinolone 16,17-acetonide, betamethasone, and fluocinolone acetonide was tested in an air pouch model. It was concluded that the air pouch model served as an application area and was used for the evaluation of commercially available corticosteroids [22].

1970: An ascitic variant of an induced hepatoma cell line was established. The original tumor was inoculated inside an air pouch. Tumor cells grew attached to the pouch walls, and in addition some cells accumulated in the fluid inside the pouch. The fluid was extracted and injected intraperitoneally in female mice and maintained for more than six passes. This method produces high amount of tumor cells free of non-tumor cells and connective tissue [23].

1973: Sensitivity of the capillary endothelium to radiation was tested. Briefly, an air pouch was induced in rats, then the area was depleted of blood vessels by freezing, and vascular proliferation was later induced employing uric acid and lithium lactate. The area becomes revascularized within 12 days. It was observed that in irradiated preparations, revascularization decreased in a dose-dependent manner [24].

1974: On the 30th symposium of the Society for Developmental Biology, the air pouch model was listed as one of the bioassays to evaluate the tumor angiogenesis factor [25].

1974: The air pouch model was used to study the binding of carcinogen 7,12-dimethylbenz[a] anthracene (DMBA) to the DNA of the mouse epidermis. The results showed that it is possible to study molecular events, such as the binding of DMBA to replicative or non-replicative DNA in vivo. It was concluded that DMBA binds preferentially to non-replicating DNA [26].

1974: An air pouch in the rat was the technique used to demonstrate that tumors can induce endothelial mitosis from 3 to 5 mm distance [27].

1974: Bladder fragments and air were subcutaneously injected in the backs of Wistar rats; within a month a cyst completely lined by urothelial cells was formed and persisted for at

least 6 months. This heterotypic bladder in the rat was described by the authors as the first step to study the implantation, progression, and spread of bladder cancer [28].

1974: The healing of tissues damaged by X-ray radiation was studied using the air pouch model. The measurement of radiation damaged was based on the DNA content of the granuloma tissue and the volume of blood produced by angiogenesis and by microcolony count. It was concluded that a single high dose of local radiation (2000–4000 rads) destroys the inner layer of rapidly growing cells, while outer layers of differentiated cells show less damage [29].

1976: Differences between the early and late stages of inflammation were studied in a carrageenan inflammation air pouch model. The results showed that vascular permeability is increased by histamine injection on the early stage, while prostaglandins increase vascular permeability on the chronic stage [30].

1976: Hypersensitivity to tobacco was studied in a mouse model. To determine the involvement of mast cell response, an air pouch was induced on the back of mice, tobacco extract was administered into the pouch, and the tissue was examined microscopically to determine the presence of degranulated and non-degranulated mast cells. It was concluded that mice serve as a good model to study tobacco hypersensitivity and that mast cell is sensitized by tobacco-specific IgG and IgE [31].

1977: The air pouch model with carrageenan was used to evaluate the effect of flurbiprofen, a nonsteroidal anti-inflammatory drug, on prostaglandin production and leucocyte migration. It was concluded that optimal doses of flurbiprofen may inhibit prostaglandin synthesis and polymorphonuclear leucocyte migration into the site of inflammation [32].

1979: It was observed that oxygenation of tumor tissues occurred after X-ray radiation in some tumors; this phenomenon was termed reoxygenation, and it was believed to affect cancer cell survival. Reinhold, Blachiewicz, and Berg-Blok modified the air pouch model to obtain a "sandwich chamber" on the back of rats, to evaluate the oxygenation effect on tumors with or without the previous radiation. They concluded that oxygen levels in the tumor microenvironment of rhabdomyosarcoma BA 1112 can temporarily increase after 20 Gy radiation [33].

1980: N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG) and benzo[a]pyrene (BP) were administered into air pouches of adult male rats. Fort-eight hours later, the granulation tissue was excised, and the density of mutated cells resistant to 6-thioguanine was determined in vitro. There was a dose-dependent increase in the mutation rate for both compounds [34].

1980: L. van den Boocaard studied the antitumor effect of heat and irradiation combined. He induced an air pouch on the back of rats and inoculated tumor cells inside the pouch. Then, the dorsal skin of the rat was immersed in a water bath at 42°C, and the air pouch prevented the excessive heating of the body, while tumor was about the same as the water bath. Tumor heating was performed for 2 hours 1 day before irradiation. L. van den Boocaard concluded that the combined therapy induced tumor regression and a longer tumor-free interval [35].

1982: An air pouch was formed on the back of rats to induce rapidly proliferating granuloma tissue. Two days later the test compounds, 5-bromo-2′-deoxyuridine (BrdU), mitomycin C (MMC), colchicine, and cyclophosphamide (CP), were administered inside the pouch;

24 hours later, the tissue was excised, and sister chromatid exchange frequencies were determined. It was concluded that mitomycin C and cyclophosphamide induce sister chromatid exchange [36].

1983: The carcinogenic potential of procarbazine was evaluated in an air pouch model in rats. It was concluded that high doses of procarbazine (300 mg/Kg) injected into the subcutaneous tissue do not induce tumors, unless an inflammatory environment had been previously established with a croton oil injection [37].

1984: An air pouch model was induced beneath the mammary fat pad of Wistar rats and injected with 20 milligrams of DMBA (7,12-dimethylbenz[a]anthracene). This technique allowed the induction of localized, transplantable, and estrogen-dependent adenocarcinomas in 67–80 days as compared to 150–180 days required by other techniques. The authors suggested that this model might be useful to study the biochemical mechanisms involved in estrogen-dependent mammary cancers [38].

1986: Sedgwick and Lees compared three different acute inflammation models: a 6-day air pouch, polyester sponge, and pleurisy. Overall, they concluded that the air pouch model was the most sensitive to assess the effect of steroids [39].

1987: Pretreatment of mice with methotrexate inhibits neutrophil chemotaxis induced by B4 and the complement protein C5a administration inside an air pouch. These results indicated that the anti-inflammatory activity of methotrexate observed in clinical trials with arthritis patients may be due to neutrophil chemotaxis inhibition [40].

1987: The air pouch model was used to study the effect of inflammation over pH. The author concluded that inflammation slightly decreases pH (about 0.5 units) and suggests that the pH variation could affect the effect of anesthetics [41].

1987: The effect of a fish oil-supplemented diet over inflammation and immune response was studied using a rat air pouch model. The rats were given 500 mg/kg/day of eicosapentaenoic acid and 333 mg/kg/day of docosahexaenoic acid, and control groups received water, oleic acid, or safflower oil for a period of 50 days. After this period, acute and chronic inflammation responses were induced and evaluated in an air pouch model with bovine serum albumin antigen. The results showed that the fish oil-supplemented diet decreases prostaglandin E2 and leukotriene B4 and increases leukocyte infiltration during the chronic inflammation state. No difference was observed on the volume of inflammatory exudate, the amount of protein in the exudate, and connective tissue proliferation. The authors concluded that fish oil-derived fatty acids can probably modulate the chronic inflammation phase and cellular immune response, by inhibition of leukotriene B4 and prostaglandin E2 synthesis [42].

1987: The tumor targeting the potential of liposomes encapsulating the radioisotope Ga-67 and an antibody against DLAA (Dalton's lymphoma-associated antigen) was evaluated in an air pouch model. The results showed that anti-DLAA improves Ga-67 uptake by tumor cells and that negative and neutral charge liposomes increase the accumulation of GA-67 to tumor tissue as compared to positively charged liposomes. Liposomes with Ga-67 but without anti-DLAA had minimal accumulation in tumor tissue and maximal accumulation in the liver and spleen [43].

1988: Bottomley, Griffiths, Rising, and Steward implanted a rat cartilage wrapped in cotton inside an air pouch model in mice. The authors observed loss of peptidoglycan and collagen, and formation of a granuloma, and tested different types of anti-inflammatory and antirheumatic drugs. They concluded that the air pouch model is better than other animal models to predict therapeutic efficacy in men [44].

1989: The distribution of latex microspheres into inflamed tissues and inflammatory exudate was investigated. Briefly, an air pouch model with carrageenan was induced in rats, and microspheres were administrated either orally or intravenously. The authors concluded that microspheres could be used as a vehicle for inflammation-targeted treatment [45].

1996: Appleton et al. evaluated the role of vascular endothelial growth factor in inflammation-mediated angiogenesis with the air pouch model. They concluded that the vascular endothelial growth factor may be an important regulator of angiogenesis during the inflammation process [46].

1999: The air pouch model was used to evaluate the chemopreventive activity of celecoxib and indomethacin. Briefly, hairless mice were fed with celecoxib or indomethacin, and air pouches were exposed to ultraviolet light. Celecoxib and indomethacin prevented tumor formation in 89% and 78% of the cases, respectively [47].

2000: The activation of NF-κB in the carrageenan air pouch model was studied and also the effect of dexamethasone over NF-κB activation. Results showed that NF-κB activation starts on the first day of inflammation and increases as inflammation progresses and decreases with dexamethasone treatment [48].

2000: Hooper et al. evaluated leukocyte populations, oxygen reactive species release, and phagocytosis, in response to the biomaterials poly(tetrafluoroethylene) (ePTFE), silicone, low-density polyethylene (LDPE), poly(L-lactic acid) (PLLA), poly(desaminotyrosyl-tyrosine ethyl carbonate) [poly(DTE carbonate)], and poly(desaminotyrosyl-tyrosine benzyl carbonate) [poly(DTBzl carbonate)]. Poly(DTE carbonate), ePTFE, LDPE, or poly[DTBzl carbonate increased the levels of superoxide anions inside the pouches. The authors stated that the air pouch method is a highly sensitive method to test the response of inflammatory cells to biomaterials [49].

2001: Retroviral vectors expressing a human interleukin-1 receptor antagonist and a soluble tumor necrosis factor receptor, where injected inside an air pouch model of inflammation. The pouch tissues and exudates were collected after 48 or 72 hours. Gene transfection was corroborated by PCR analysis. And, decrease of inflammation was observed in transfected mice by histological analysis. The authors concluded that the air pouch model is useful to evaluate gene therapy [50].

2004: An air pouch model was used to determine that acute neutrophilic inflammation in response to urate crystals is dependent of chemokines that bind the mIL-8RH receptor, the mice homolog of CXCR-2 chemokine 8 receptor [51].

2007: A study evaluated the antitumor effect of liposome-encapsulated anatase particles of titanium dioxide (LT). The air pouch was inoculated with NBT-II bladder cancer cells, to simulate bladder cancer, and treated with LT injections followed by UVA radiation. Tumor

growth inhibition and increased survival were observed particularly in the LT + UVA radiation group. The results suggest that LT is probably more effective than not encapsulated anatase particles of titanium dioxide for the treatment of bladder cancer [52].

2009: An air pouch model was induced to determine if interleukin-17 could initiate an inflammatory response. It was concluded that interleukin-17 cannot start an inflammatory response, but it is able to increase inflammation in its early stages [53].

2009: Kourelis et al. used the air pouch model for the evaluation of early immune response. This group determined the immunoregulatory properties of *Lactobacillus paracasei* subsp. *paracasei* B112, DC205, DC215, and DC412 strains [54].

2013: The air pouch model has also been validated for nanoparticle evaluation by Vandooren et al. This group evaluated biocompatibility, toxicity, and inflammatory and adaptive immunological response to nanoparticles designed for nanomedicine. They reported that this technique yields reproducible data, with three mice per test [55].

2014: Eteraf-Oskouei et al. evaluated the antiangiogenic effect of honey in vivo. This group induced an inflammation air pouch model in Wistar rats, by injecting 1 milliliter of carrageenan (1%) on day 6. Honey was injected into the pouch at the same time as carrageenan and then for 2 consecutive days. The evaluation parameters included hemoglobin concentration, vascular endothelial growth factors and prostaglandin E2 levels, and granulomatous tissue weight. The results showed a decrease of angiogenesis, and it was concluded that honey might be useful in the treatment of granulomatous inflammatory conditions [56].

2015: Another report published by Eteraf-Oskouei et al. evaluated the antiangiogenic effect of *Ficus carica* leaf extract. The volume of exudates, the cell number, and TNFα, PGE2, and VEGF levels contained in carrageenan air pouches were measured, and for angiogenesis determination, they measured hemoglobin quantity. The extract significantly decreased the volume of exudate and leukocyte accumulation and levels of TNFα, PGE2, and VEGF; it also inhibited angiogenesis [57].

2015: A study evaluated the role of interleukin-17 in invasive breast cancer tumor pathogenesis. Air pouches were created on the back of mice, and 4 t1 or 67NR supernatants (metastatic and nonmetastatic murine mammary cancer cell lines, respectively) were injected into the pouches. Twenty hours later cell infiltrates were harvested from the pouches and stimulated with phorbol myristate acetate (PMA) plus ionomycin and brefeldin A, and intracellular levels of interleukin-17 were determined by flow cytometry analysis. From this experiment it was concluded that interleukin-17 producing $CD3^+$ cells were significantly higher in the group treated with the 4 T1 metastatic cancer cell line supernatant [58].

2018: Our group adapted the air pouch model for the screening of antitumor properties of the bio-compound IMMUNEPOTENT CRP. We induced the air pouch model, inoculated L5178Y-R cancer cells, and determined if our compound interfered with tumor implantation. We concluded that our bio-compound has antitumor properties [59].

In our opinion the air pouch model is a valuable technique for the evaluation of compounds with antitumor and tumor-preventive and/or tumor-chemopreventive properties. In **Table 1**, we summed the main advantages and disadvantages of the air pouch model for cancer as compared to in vitro cell culture.

	In vitro	*In vivo* (air-pouch model)
Advantages	Simple Highly reproducible Human origin cells Fast result production There are no concerns regarding animal ethics	Simple Highly reproducible A large number of compounds can be assessed with minimum amount of animals Direct tumor-drug interaction Maintains tumor heterogeneity and complex interactions with immune system and stroma cells Complex tumor interactions (e.g immune cell infiltrates) and systemic parameters (e.g metastasis and pharmacokinetics) can be also be evaluated Easy tumor detachment
Disadvantages	Low correlation with *in vivo* results Artificial culture conditions (nutrient, oxygen levels, drastic pH changes, among others) No tumor cell heterogeneity No interactions with immune or stromal cells Excessive plastic use (non-ecological) Do not assesses metastasis, pharmacokinetics, and other crucial variables	There are concerns regarding animal ethics Specialized installations for animal breeding are needed Not all human targets have a murine homolog target

Table 1. Comparison between the *in vitro* and the *in vivo* air pouch model cell line culturing and drug discovery.

3. Air pouch methodology

Air pouch inflammation model.

In general, a common process to induce air pouches is performed as follows:

1. Sterile air is obtained in a laminar flow station by filtration through a Millipore filter (0.22 μm) directly into a 10 mL syringe (**Figure 2**).
2. Five milliliters of sterile air is injected subcutaneously into the shaved skin site on the back of each mouse (**Figure 3**).
3. The pouches are allowed to settle for 3 days to permit the healing of the wound. The pouch is then reinflated with 5 mL of sterile air and left for 3 more days before treatments.
4. On day 8, the pouches of the experimental groups are filled with necessary doses of the compound to test, according to our requirements.

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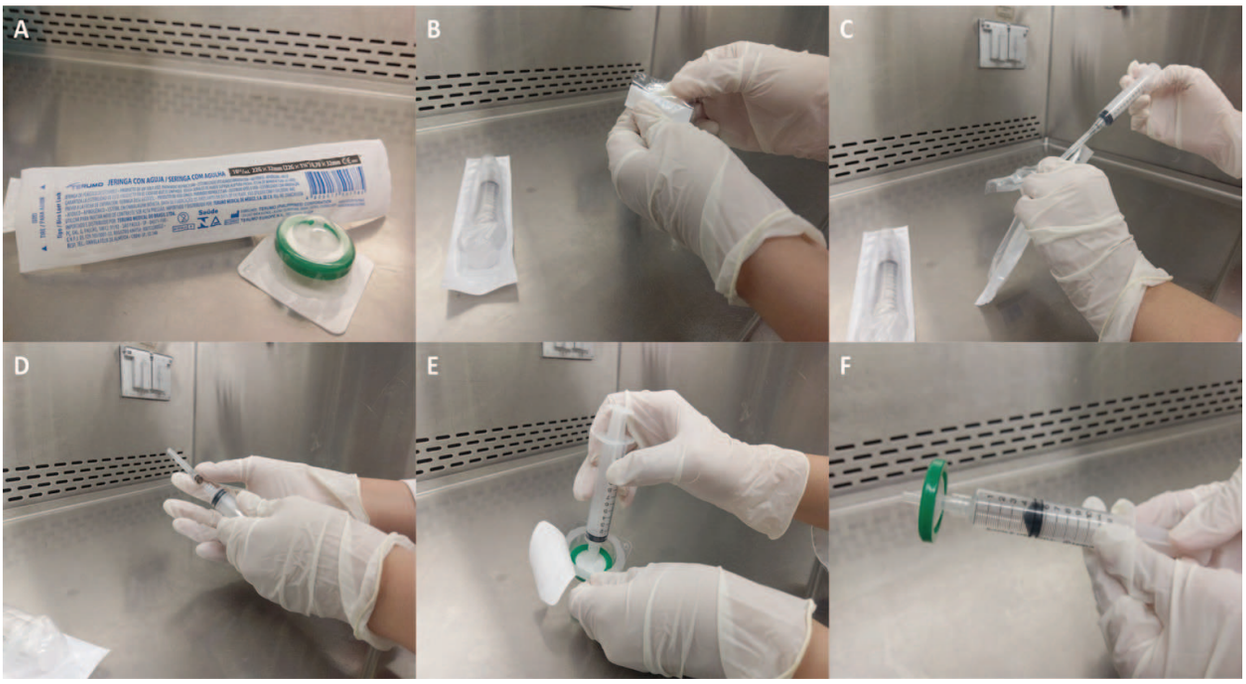

Figure 2. Step-by-step sterile air filtration in laminar flow hood. A sterile syringe that can contain 5 mL of air and a 0.2 μm filter (A) packages is opened inside a laminar flow hood (B), the syringe is carefully removed (C), the needle is detached (D), the syringe is attached to the filter (E), and 5 mL of air is loaded into the syringe (F).

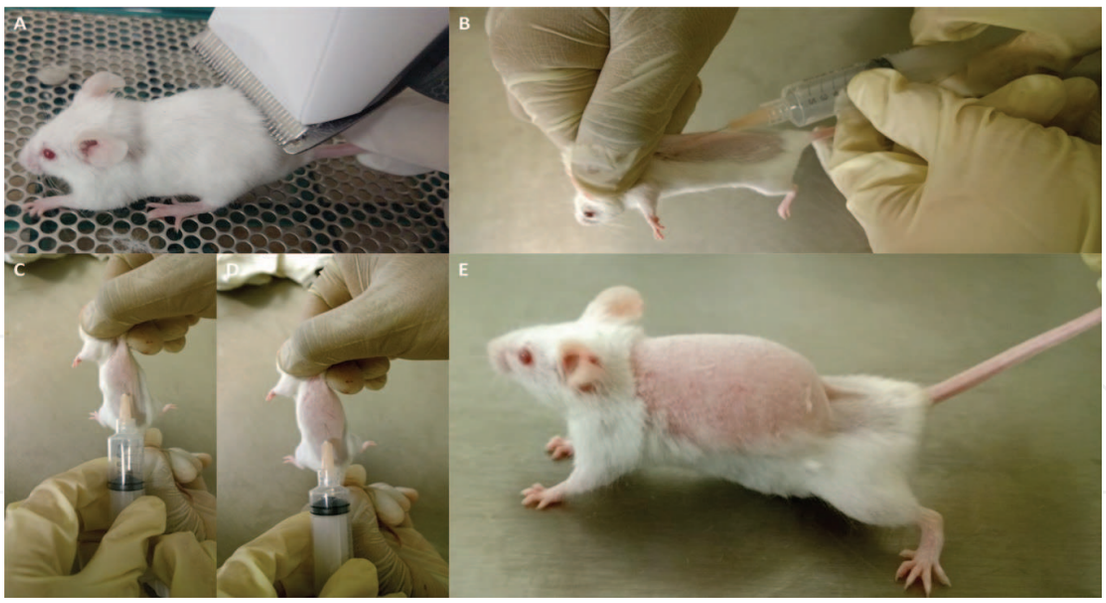

Figure 3. Air pouch formation. The rodent back is shaved (A); the animal is held, and air is injected subcutaneously into the back (B); and air pouch inflation is observed (C and D). The air pouch completely formed (E).

Note: Inflammatory agents commonly used to induce the inflammation air pouch model are ultraviolet radiation, 12-o-tetracanoilphorbol-13-acetate, oxazolone in acetone, turpentine, carrageenan, brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, aerosil, implantation of pellets of compressed cotton, collagen, incomplete Freund's adjuvant, and papaya latex [60].

Air pouch modified for tumor induction

1. Inside the laminar flow hood, 5 mL of air is charged into a sterile syringe through a 0.2 μm filter (to avoid any contaminant particles) (**Figure 2**).
2. The animal back is shaved and sprayed with alcohol (70%). The animal is hold by the scruff and tail (as shown in **Figure 2B**), and the sterile air is injected subcutaneously using a 21G × 1 ¼ caliber needle. The formation of the air pouch is immediately observed (**Figure 3**).
3. After 72 hours the procedure is repeated once again to avoid disinflation. Tumor cells are inoculated 48 hours after the air pouch induction (**Figure 4A**).

Protocol 1: Air pouch model for tumor implantation

1. Viable tumor cells in 100 μL of phosphate-buffered saline are injected subcutaneously into the air pouch using a 30G × 1/2 caliber needle. Cell number varies depending on the cell line.
2. Treatment administration begins 24 hours later. Treatment administration scheme depends on the tested drug. We recommend adding a group treated with placebo (injectable solution) and with the reported treatment (e.g., chemotherapy), as positive and negative tumor growth controls, respectively.
3. For therapy evaluation, mice are sacrificed after 9 days of treatment, and tumor is resected (**Figure 4B**) and can be stained with hematoxylin and eosin (**Figure 4C**).

Protocol 2: Air pouch model for tumor progression

1. Viable tumor cells in 100 μL of phosphate-buffered saline are injected subcutaneously into the air pouch using a 30G × 1/2 caliber needle. Cell number varies depending on the cell line.
2. Treatment administration begins 7 days after tumor inoculation (days can vary depending on tumor growth characteristics). Treatment administration scheme depends on the tested drug. We recommend adding a group treated with placebo (injectable solution) and with the reported treatment group (e.g., chemotherapy), as positive and negative tumor growth controls, respectively.
3. For therapy evaluation, mice are sacrificed after 21 days of treatment.

Note: For each experiment design, it is important to take into consideration variables that could alter the results, such as animal age and sex, and treatment administration.

Animal age: A study performed by Jackson et al. revealed that there is a discrepancy on the age of rodents used for neuroscience, immunology, cancer, genetics, physiology, and toxicology research. The age can vary from 2 to 160 weeks, and 8 to 12 weeks old are the most used.

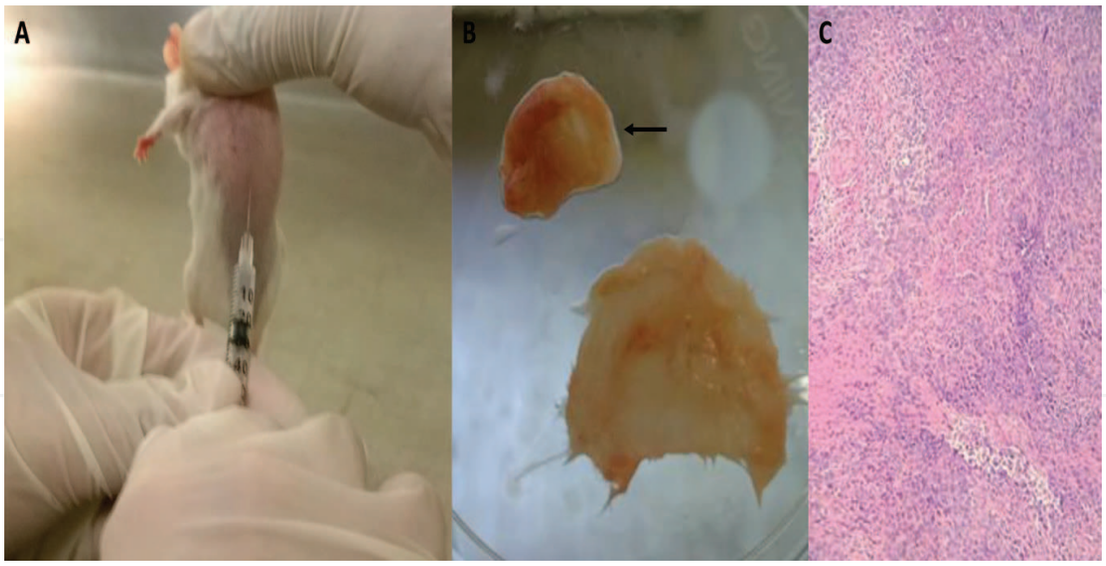

Figure 4. Tumor cell inoculation into the air pouch. 1×10^6 viable 4 T1 cells in 100 μL of PBS were inoculated into an air pouch (A). The mouse was sacrificed 12 days after for tumor evaluation. The skin from the air pouch (B) and tumor (B) were detached (the arrow points to the tumor). Tumor sections were stained with hematoxylin and eosin (C).

The appropriate age range of the rodents depends on the experiment and relevant age for human disease. For example, 3- to 6-month C57BL6/J mouse is comparable with 20–30 human years, 10–14 months compares to 38–47 human years, and 18–24 months compares to 56–69 human years [61].

Animal sex: This can also affect the therapy outcome. This is particularly true for targeted therapies, and it has become a requirement for any applications for the National Institutes of Health (NIH) to report the male and female ratio in preclinical studies [62]. Genes encoded by the Y chromosome include inflammatory pathway genes, while X chromosome encodes genes for Toll-like receptors, cytokine receptors, and transcriptional and translational regulator genes. Although X chromosome is present in both sexes, female X polymorphism allows for mosaicism and randomly silenced alleles. It has been observed that male sex leads to data alteration in pharmacology and neuroscience, and female sex affects immunology results [62, 63]. The National Institutes of Health (NIH) announced in May 2014 that they "require applicants to report their plans for the balance of male and female cells and animals in preclinical studies in all future applications" [62].

Therapy administration: Carcinogenesis is described as a three-stage process, initiation, promotion, and progression. Compounds that prevent or delay any stage of carcinogenesis are called chemopreventive agents. Chemoprevention is defined as the use of compounds to reduce the risk or delay the development of cancer or to avoid its recurrence. Bio-compounds such as food-derived polyphenols, usually inhibit initiation and promotion, of induced cancer, and therefore are considered chemopreventive agents [64]. When testing antitumor activity of natural or synthetic compounds, highly variable results can be obtained based on the type of tumor, the administration dose and frequency, and the evaluation parameters: tumor incidence, growth, or metastasis. Therefore, the purpose and parameters of the preclinical testing must be clearly defined [65].

4. Suggested evaluation parameters

Angiogenesis: Angiogenesis is a physiological process that refers to new blood vessel formation. In the tumor microenvironment, angiogenesis is over-induced to sustain tumor growth and metastasis. The vascular endothelial factor is one of the parameters used to evaluate angiogenesis. This factor induces angiogenesis, and it is increased in tumor and tumor-adjacent stroma tissue and correlates with tumor aggressiveness and with the patient prognosis [66]. Another parameter for angiogenesis evaluation is the tissue hemoglobin content [56, 57]. Angiogenesis can be measured in the tumor and adjacent tissue (skin from the air pouch), with the following techniques: flow cytometry, fluorescence microscopy, Western blot, immunohistochemistry, ELISA, and RT-PCR.

Cancer-associated fibroblasts (CAFs): Fibroblasts are the most abundant cells of the connective tissue; they produce collagen for the extracellular matrix and are involved in wound healing. In the tumor microenvironment (TME), cancer cells and other stroma cells induce fibroblasts to produce tumor-promoting substances such as epidermal and hepatocyte growth factors; chemokines CXCL12, CXCL14, and CCL5 [that attract immature and suppressor immune cells]; vascular endothelial growth factor; and interleukin-6, among others. This type of fibroblasts is termed activated or cancer-associated fibroblasts (CAFs), and they correlate with tumor growth, progression, metastasis, and chemoresistance [67].

Several cell markers are used to detect CAFs, including fibroblast activation protein α, podoplanin-a, S100A4, vimentin, fibroblast-specific protein-1, platelet-derived growth factor receptors α and β, and insulin-like growth factor-binding protein [67]. CAFs can be measured in the tumor tissue with the following techniques: flow cytometry, fluorescence microscopy, Western blot, immunohistochemistry, ELISA, and RT-PCR.

Indoleamine 2,3-dioxygenase 1 (IDO1): It is a cytosolic enzyme that catabolizes tryptophan into kynurenine, a metabolite with immunosuppressive properties. IDO1 is overexpressed in more than 50% of all tumors. Increased levels of IDO1 correlate with the decrease of natural killers and specific effector T cells and increase of regulatory T cells, tolerogenic dendritic cells, and myeloid-derived suppressor cells. IDO1 also correlates with tumor progression and multidrug resistance. It is therefore considered a tumor progression biomarker and a promising therapeutic target [68]. IDO levels can be measured in the tumor tissue with the following techniques: flow cytometry, fluorescence microscopy, Western blot, immunohistochemistry, ELISA, and RT-PCR.

Interferon gamma (IFN-γ): It is a cytokine produced by natural killer cells, natural killer T cells, antigen-specific CD4 Th1 and CD8 cytotoxic effector lymphocytes, non-cytotoxic innate lymphoid cells, and mucosal epithelial cells. IFN-γ induces class I and II major histocompatibility complex expression on antigen-presenting cells, promotes natural killer activity, increases antigen presentation and lysosome activity of macrophages, activates inducible nitric oxide synthase, induces production of IgG by plasma cells, promotes adhesion required for leukocyte migration, and has direct antiviral effect (by induction of tripartite motif-containing protein 5 and apolipoprotein B-mRNA editing enzyme, among others) [69]. IFN-γ levels correlate

with good prognosis of patients with different types of cancer [69, 70]. IFN-γ levels can be measured in the tumor tissue or tumor tissue supernatant with the following techniques: flow cytometry, ELISA, and RT-PCR.

Lipid rafts: Cholesterol and sphingolipids form specific domains termed lipid rafts that regulate receptor-ligand interactions. In cancer cells, signaling protein and pro-oncogenic receptor activation correlates with their location inside the lipid rafts; disruption of lipid rafts induces apoptosis in cancer cell lines. Lipid rafts are characterized by the presence of glycosylphosphatidylinositol (GPI)-anchored proteins [71]. Also, acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), ATP citrate lyase (ACLY), and other lipogenic enzymes that promote cholesterol synthesis are altered in most tumors [72]. Lipid rafts can be measured in the tumor and adjacent tissue (skin from the air pouch), with the following techniques: flow cytometry, fluorescence microscopy, Western blot, immunohistochemistry, and ELISA.

Liver toxicity: Synthetic and biological compounds are often metabolized and excreted by the liver. Certain drugs can be metabolized to reactive compounds that bind to intracellular proteins inducing oxidative stress and cell death. Liver toxicity must be evaluated in early phase or preclinical studies of any drug and can be assessed by transcriptomics, cellular respiration, ATP (adenosine triphosphate), ROS (reactive oxygen species), covalent binding, apoptosis or necrosis, and bile salt export pump inhibition tests [73]. Liver toxicity can be evaluated in the liver tissue by immunohistochemistry or Western blot or in peripheral blood by flow cytometry or ELISA. Furthermore, blood tests can be included (glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, alkaline phosphatase).

Mesenchymal stem cells (MSCs): Mesenchymal stem cells are multipotent stem cells characterized by CD73, CD105, and CD90 surface markers. MSCs have the potential to differentiate into osteoblasts, chondrocytes, and adipocytes and are recruited to injured tissues for healing. However, under different stimuli in the TME, MSCs secrete PGE_2, IL-6, IL-10, IL-17b, EGF, and CCL5, therefore promoting cancer stemness and metastasis; furthermore, they have been shown to regulate cancer cell metabolism by exosome secretion [74]. MSCs can be evaluated in the tumor tissue with the following techniques: flow cytometry, fluorescence microscopy, Western blot, and immunohistochemistry.

Tumor-associated macrophages: Macrophages are specialized phagocytic cells of the immune system. In response to various stimuli, macrophages shift their phenotype to M1 or M2. M1 macrophages are associated to an inflammatory response with antitumor properties; on the other hand, M2 macrophages promote tumor growth and have anti-inflammatory properties. Tumor-associated macrophages (TAMs) found in the TME resemble M2 macrophages and correlate with poor prognosis. TAMs produce IL-23, IL-17, IL-6, PGE2, IL-10, and indoleamine 2,3-dioxygenase and CCL17, CCL18, and CCL22, which are chemotactic factors for regulatory T cells. TAMs are characterized by CD163, CD204, or CD206 surface markers [74, 75]. TAMs can be evaluated in the tumor tissue (skin from the air pouch), with the following techniques: flow cytometry, fluorescence microscopy, Western blot, and immunohistochemistry.

Tumor growth rate: Tumor size is defined by the Response Evaluation Criteria in Solid Tumors (RECIST) as the sum of the longest diameters of the tumor mass; to report the tumor growth rate, tumor size can be measured during relevant therapy time points, for example, before treatment, after the first treatment cycle, after the last cycle of treatment, and after discontinuation of the treatment [76]. Also, the Ki67 levels, a protein in all phases of the cell cycle (G1, S, G2, and mitosis), except for the resting phase (G0), often correlate with tumor growth and progression [77]. Tumor growth can be measured with a caliper (volume), or weighted after removal, or determination of Ki67 levels in the tumor tissue by flow cytometry, fluorescence microscopy, Western blot, and immunohistochemistry.

5. Conclusion

To be clinically successful, an anticancer drug must have effect over cancer cells in the tumor microenvironment context; however, **in vitro** models do not include all of its components. Therefore, the improvement of cancer models is considered a priority for the current drug development [55].

In the current chapter, we propose a modified air pouch model as an alternative or complement to **in vitro** studies. As previously described, the air pouch model has been extensively used to evaluate inflammation process, anti-inflammatory compounds, immune response, biomaterial compatibility, and of course cancer development and treatment.

The air pouch model allows the administration of higher volumes of chemotherapy alone or combined with other treatment modalities, including targeted therapy, immunotherapy, and biological compounds, to determine single or cumulative antitumor effects, simulating the true clinical condition and treatment.

Conflict of interest

The authors declare that there is no conflict of interest.

Notes/thanks/other declarations

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List of abbreviations

poly(DTBzl carbonate)	poly(desaminotyrosyl-tyrosine benzyl carbonate)
poly(DTE carbonate)	poly(desaminotyrosyl-tyrosine ethyl carbonate)

ACC	acetyl-CoA carboxylase
ACLY	ATP citrate lyase
ATP	adenosine triphosphate
BrdU	5-Bromo-2′-deoxyuridine
CAFs	cancer-associated fibroblasts
CP	cyclophosphamide
DMBA	Dalton's lymphoma-associated antigen
ELISA	enzyme-linked immunosorbent assay
ePTFE	polytetrafluoroethylene
FASN	fatty acid synthase
GPI	glycosylphosphatidylinositol
LDPE	low-density polyethylene
MMC	mitomycin C
PLLA	poly(L-lactic acid)
PMA	phorbol myristate acetate
RECIST	response evaluation criteria in solid tumors
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
TAMs	tumor-associated macrophages
TME	tumor microenvironment

Author details

Moisés Armides Franco-Molina*, Silvia Elena Santana-Krímskaya and Cristina Rodríguez-Padilla

*Address all correspondence to: moyfranco@gmail.com

Laboratory of Immunology and Virology of the Faculty of Biological Sciences of the Autonomous University of Nuevo León, San Nicolás de los Garza, México

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