

## A new therapeutic proposal for inoperable osteosarcoma: Photodynamic therapy

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### ABSTRACT

**Background:** Osteosarcoma, a malignant tumor characterized by bone or osteoid formation, is the second most common primary bone neoplasm. Clinical symptoms include local and surrounding pain, unrelieved by rest or anesthesia. Osteosarcoma has a poor chemotherapeutic response with prognosis dependent on complete tumor excision. Therefore, for inoperable osteosarcoma new therapeutic strategies are needed. The present study aimed to develop murine models of cranial and vertebral osteosarcoma that facilitate simple clinical monitoring and real-time imaging to evaluate the outcome of photodynamic therapy based on a previously developed photosensitizer.

**Methods:** Balb/c nude mice were divided into two groups: the cranial and vertebral osteosarcoma groups. Each group was further subdivided into the photodynamic therapy-treated and untreated groups. Images were obtained by scintigraphy with <sup>99m</sup>Tc-MIBI and radiography. Tumor growth, necrotic area, osteoid matrix area, and inflammatory infiltration were analyzed.

**Results:** Cranial and vertebral tumors could be macroscopically observed and measured. Radiographic and scintigraphic images showed tumor cells present at the inoculation sites. After photodynamic therapy, scintigraphy showed lower tumoral radiopharmaceutical uptake, which correlated histologically with increased necrosis. Osteoid matrix volume increased, and tumor size decreased in all photodynamic therapy-treated animals.

**Conclusion:** Cranial and vertebral osteosarcoma models in athymic mice are feasible and facilitate *in vivo* monitoring for the development of new therapies. Photodynamic therapy is a potential antitumoral treatment for surgically inoperable osteosarcoma.

### 1. Introduction

Osteosarcoma is the second most common primary bone malignancy characterized by osteoid production [1]. Osteosarcoma is one of the most common cancers in childhood, manifesting as leukemia, brain, and other nervous system cancers and occurring predominantly after the first decade of life.

Despite a plethora of diagnostic and therapeutic modalities, curative option for osteosarcoma is the association of multiagent chemotherapy with surgery. The surgical procedure adopted depends on the primary anatomical site and extent of tumor invasion. In osteosarcoma,

incomplete tumor removal is associated with local recurrence [2,3].

Chemotherapy or surgery alone is not sufficient for primary tumor or metastasis control [2,3]. Therefore, for patients with primary tumors or metastases at sites where excision may be contraindicated, such as the jaw, skull, or spine, there are few effective therapeutic options. The development of new therapeutic strategies for these cases is of paramount importance.

There are few animal models of osteosarcoma. Initially, osteosarcoma models were developed by exposing experimental animals to high-dose internal or external radiation [4–7], benzopyrene and cholesterol in the tibia [8], or oncogenic viruses [9]. More recently, due to

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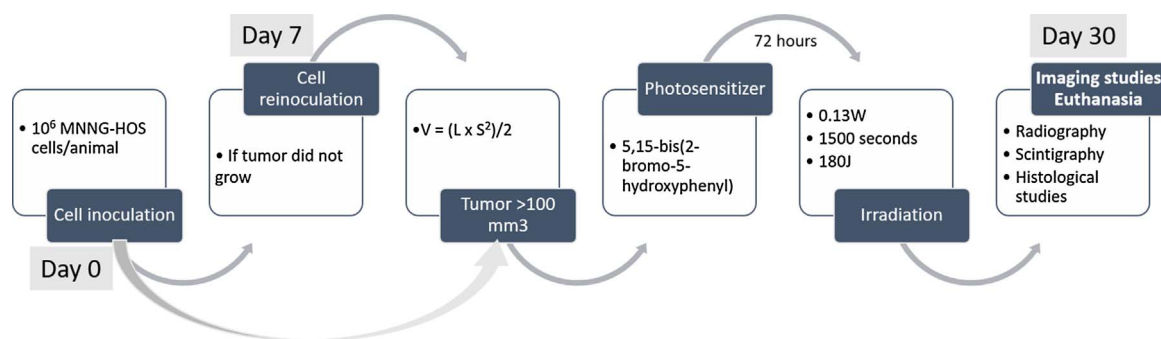


Fig. 1. Procedures timeline. Animals were injected with OS cells on day 0; when the tumor did not develop up to day 7, cell reinoculation was performed; the photosensitizer was administered when tumor reached  $100 \text{ mm}^3$  and irradiation was performed 72 h after photosensitizer administration; animals were euthanized for histological analysis on day 30, after radiography and nuclear medicine imaging.

the unpredictability of these models and the desire for more robust models, new studies reporting the spontaneous occurrence of osteosarcoma in dogs [10] and rats [11] have emerged.

In 2001, Khanna et al. [12] developed an orthotopic xenograft model in the tibia of Balb/c nude. Rat osteosarcoma cells (K7M2) were injected into the proximal tibia, resulting in more successful primary tumor growth and an increased incidence of pulmonary metastases compared with subcutaneous, intravenous, or intramuscular injection. At the same time, the rat cell line OS/UMR 106-01 was used to establish an animal model in athymic mice [13]. Several attempts to develop lung metastases after inoculation of human osteosarcoma cells (HOS cell line) *in vivo* have been described, in both heterotopic and orthotopic sites, but without success [14–16]. However, the development of metastases was possible after HOS cell line modification using N-methyl-N'-nitro-N-nitrosoguanidine [17]. A later successful technique utilized N-methyl-N'-nitro-N-nitrosoguanidine-HOS cell (MNNG-HOS) inoculation in the open tibial cortex, as opposed to using bone cortex transfixion [18].

Despite existing osteosarcoma models, there are no reports of cranial or vertebral osteosarcoma models. In addition, studies evaluating alternative treatment approaches for vertebral or cranial osteosarcoma have not been conducted.

Photodynamic therapy, uses photosensitizers activated by visible light, to kill cancer cells. The irradiation wavelength range should be selected according to the absorption spectrum of the photosensitizer and consider that longer wavelengths are capable to reach further within the tissues. Photosensitizing agents can be administered intravenously, orally, or topically. Agents are taken up by the cancer cells and light is applied to the area to be treated. Mechanistically, photodynamic therapy decreases tumor cell viability through activation of stress response pathways and cell death programs [20]. In addition, photodynamic therapy causes tumor vasculature collapse to restrict oxygen and nutrient supplies [21] and induces an immune response [22–25].

Because it is relatively safe and elicits significant tumor growth inhibition, photodynamic therapy could be a promising cancer treatment [19,20,21]. Previous studies in heterotopic osteosarcoma models [26] and *in vitro* [27] support this expectation. Photodynamic therapy has numerous advantages compared with surgery and other treatments. Photodynamic therapy is a minimally invasive short duration procedure that can be repeated without causing significant scarring and it allows precise targeting of the lesion [20,21]. Furthermore, photodynamic therapy costing is favorable and does not have long-term side effects [20,21,26,27]. Therefore, photodynamic therapy might represent a potentially efficacious therapy for surgically inoperable osteosarcoma.

To date, studies evaluating the efficacy of photodynamic therapy for cranial or vertebral osteosarcoma have not been conducted. The present study aimed to develop novel murine models of human cranial and vertebral osteosarcoma that would allow real-time observation and

monitoring of photodynamic therapy as a potential osteosarcoma treatment.

## 2. Material and methods

### 2.1. Cell culture

MNNG-HOS cells (CRL-1547) were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). Cells were propagated in Dulbecco's Modified Eagle Medium (Sigma, Sigma-Aldrich, Inc, USA) supplemented with  $100 \mu\text{M}$  sodium pyruvate (Gibco ThermoFisher Scientific, United Kingdom), 10% fetal bovine serum (Gibco, ThermoFisher Scientific, South America), and 1% antibiotic ( $100 \text{ U/ml}$  penicillin and  $10 \mu\text{g/ml}$  streptomycin; Gibco ThermoFisher Scientific, Netherlands) at  $37^\circ\text{C}$  in a humidified chamber aerated with 5% carbon dioxide. Culture medium was replaced every 48 h.

After propagation, cells were separated by incubation in 3 ml 0.25% trypsin-ethylenediaminetetraacetic acid (Gibco, ThermoFisher Scientific, USA) for 3 min. Cell viability was determined by evaluating trypan blue exclusion in a Neubauer chamber.

### 2.2. Osteosarcoma orthotopic animal models establishment

Six to eight-weeks-old Balb/c nude mice weighing 18–25 g were purchased from Charles River Laboratories (Barcelona, Spain). All experiments (Fig. 1) were conducted in accordance with the Declaration of Helsinki, the Portuguese Society of Animal Science Laboratory guidelines [28] according to the Council for International Organization of Medical Sciences Ethical Code for Animal Experimentation, and the Ethical Committee and Research São Francisco University" (Number 001.05.12).

Animals were housed in temperature-controlled cabinets, over a 12 h light-dark cycle, with ad libitum access to food and water. All procedures were performed in a flow cabinet to ensure an aseptic environment.

For cancer cell inoculation, before light irradiation, and for euthanasia mice were anesthetized by subcutaneous administration of a solution of 77% ketamine (Ketalar®, Parke-Davis, Portugal) and 23% chlorpromazine (Largactil®, Victoria Laboratories, Portugal). Cell reinoculation was only performed if a tumor did not develop.

OS cells cultured were suspended in  $50 \mu\text{l}$  of normal saline and implanted by percutaneous puncture using 1 ml syringes and 30 gauge hypodermic needles ( $0.3 \times 13 \text{ mm}$ ) in both models, vertebral and cranial osteosarcoma.

#### 2.2.1. Cranial osteosarcoma model

For the cranial model ( $n = 12$ ), MNNG-HOS cells ( $10^6$  cells/animal) were implanted by percutaneous puncture at a  $45^\circ$  angle. Bone scarification was performed using the bevel of the hypodermic needle,

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