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Effective antiproliferative effect of meloxicam on prostate cancer cells: Development of a new controlled release system

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ABSTRACT

Recent studies have shown that COX-2 inhibitors, such as meloxicam, have demonstrated promising results when used with chemotherapy. Based on these findings, this is the first study in which the antiproliferative effect of meloxicam is investigated on two prostate cancer cell lines (PC3 and DU-145). We have also evaluated if this antiproliferative effect is dose- and/or time-dependent. Meloxicam is assayed at a concentration range of 10–800 μM for 24, 48 and 72 h. Our results reveal that meloxicam has a selective dose- and time-dependent antiproliferative effect against PC3 but not against DU-145 cells. In PC3 cells the IC_{50} decreased from 740 μM at 24 h to 515 μM at 72 h after meloxicam treatment. Chemoembolization based on microspheres has been emerged as a novel and promising way for antitumoural therapy; therefore, in our study we have developed and characterized a new controlled release system consisting of biodegradable PLGA/PEG-derivative meloxicam microspheres. The optimized formulation has a mean particle size of 13.06 ± 0.09 μm, mean encapsulation efficiency of 58.44 ± 4.53% and releases 0.45 ± 0.05 μg meloxicam/day/mg microspheres between days 3 and 28 of the in vitro release assay. In conclusion, we should consider meloxicam as a possible adjuvant agent in the treatment of prostate cancer.

1. Introduction

Prostate cancer (PCa) is presently the second leading cause of cancer-related death and the most commonly diagnosed non-skin cancer in men. Diagnosis and treatment of PCa has had many advances in the past 20 years; unfortunately, the existing treatment approaches and surgical intervention have been proven to be inadequate for the management of the disease (Han et al., 2008a,b). In addition to date, no effective therapeutic treatment allows the abrogation of its progression to more invasive disease forms. In fact, while PCa is frequently curable in its early stage, however approximately one-third of PCa patients with advanced disease require development of novel therapeutic approaches for its control and to improve patient survival.

Many studies have focused on the identification of the molecular mechanisms of development and progression of PCa, which are complicated and likely to involve multiple factors, such as tumour suppressor genes, oncogenes, growth factors, adhesion molecules and angiogenesis.

Cyclooxygenase (COX), the enzyme required for the conversion of arachidonic acid into prostaglandins, plays a possible role in oncogenesis (Fosslien, 2000). Although three isoforms of cyclooxygenase are known until now (COX-1, COX-2 and COX-3), there are only evidences of an important role of COX-2 in carcinogenesis. COX-2 activity increases during inflammation processes and also, it is overexpressed in many types of malignant tumours, such as colorectal, prostate, breast, liver, pancreatic primary tumours and lung cancer, with pharmacological studies suggesting that it is a useful therapeutic target (Naruse et al., 2006; Gupta et al., 2000). Therefore, inhibition of COX-2 enzyme could be used as a strategy against carcinogenesis progression. Some authors have suggested the use of COX-2 inhibitors in anticancer therapy combined with other chemotherapeutic agents (Gasparini et al., 2003). For instance, this potential is currently under investigation by several authors (Falandry et al., 2009; Suzuki et al., 2009).

Meloxicam is a preferential COX-2 inhibitor which has proven to inhibit cell proliferation of different cancer cell cultures, such as colorectal cells (Goldman et al., 1998), non-small lung cancer cells (Tsubouchi et al., 2000) and osteosarcoma cells (Wolfesberger et al., 2006), as well as in animal tumours (Tsuchida et al., 2005; Kern et al., 2004). Nevertheless, some authors have reported that inhibition of cell proliferation by meloxicam depends on the concentration used and could be significantly different between cell

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lines (Wolfesberger et al., 2006). Moreover, to date the antiproliferative effect of meloxicam has not yet been demonstrated in many other cancer cell lines. For this reason it is necessary to study the behaviour of cancer cells in contact with meloxicam in order to consider its use as an adjuvant agent for the treatment of other types of cancer, such as prostate cancer. To fulfil this objective, in this work we have studied the antiproliferative effect of meloxicam on two PCa cell lines of different metastatic potential (PC3 and DU-145), taking into consideration that PCa cells with high- and low-metastatic potential vary in their biological properties, such as proliferation, adhesiveness, invasiveness, and motility (Graff et al., 2001; Nelson et al., 2003; Liao et al., 2006; Wang et al., 2006).

To be able to achieve enough effective intratumoural concentrations of meloxicam in PCa, high doses of the drug are needed when administered in a conventional dosage form thereby resulting in compromising adverse systemic side-effects; therefore, in our study a new controlled release system consisting of PLGA/PEG-derivative microspheres of meloxicam has been developed and characterized. This new therapeutic system will allow maximizing the efficiency of the drug after direct administration near the tumour by means of chemoembolization transcatheter prostatic arterial administration. Embolizations have been extensively used to occlude vessels in the last few decades (Rafael et al., 2002), and more recently these techniques have been employed more broadly with greater precision and convenience (Bendszus et al., 2000; Yamamoto et al., 2003). Administration of the new controlled release system by artery embolization could maintain higher intratumoural drug concentrations for a longer period of time, reducing drug levels of body circulation, thereby achieving the goal of targeted tumour therapy. The new controlled release system that we have developed was prepared with PLGA, a polymer that offers the advantage of its biodegradation, being able to disappear gradually while releasing the drug from the site of action. The new formulation is also characterized and drug release optimized by means of the addition of a biodegradable oil (PEG-derivative) which allowed us to better modulate the release rate of meloxicam from the microspheres (Gao et al., 1995; Fernández-Carballido et al., 2008).

2. Materials and methods

2.1. Materials

Meloxicam was supplied by Sigma–Aldrich Chemical (Madrid, Spain). PLGA 50:50 poly(D,L-lactide-co-glycolide) (Resomer® RG 503, Mw = 34 kDa (GPC)) was obtained from Boehringer Ingelheim (Ingelheim, Germany). PEG-derivative (Labrafil® M 1944 CS) Gattefossé (Saint-Priest, France) is a mixture of mono-, di- and triglycerides and mono- and di-fatty esters of polyethylene glycol 300, oleic acid being the predominant fatty acid. Polyvinyl alcohol (PVA) (Mw = 49 kDa) was obtained from Sigma–Aldrich Chemical (Madrid, Spain). All other reagents were of analytical grade and provided by Merck (Darmstadt, Germany). Distilled and deionized water (Milli-Q, Millipore Corporation, Bedford, MA, USA) was used in the preparation of all buffers and solutions.

2.2. Cytotoxicity of meloxicam in fibroblasts

In order to evaluate the cytotoxic effect of meloxicam on non-tumoural cells, a flow cytometry analysis was performed. Cytotoxicity was tested on cell line NIH-3T3 obtained from American Type Culture Collection (ATCC, Manassaa, VA, USA). Cultures were maintained at 37 °C under 5% CO_2 and 95% air atmosphere. Cells were detached from flasks and placed into petri dishes at a density of 500,000 cells/petri dish in RPMI-1640 medium (Invitrogen, Spain). Cells were treated with different concentrations of meloxicam (10, 25, 50, 100, 200 and 400 μM). For this, a stock solution of meloxicam was prepared using a fixed amount of DMSO and then added to the culture medium to obtain the final drug concentrations. All solutions were sterilized prior to use.

After 48 h of treatment, cell viability was evaluated using propidium iodide (PI) (Invitrogen, Spain) for 30 min. Samples were analyzed by a flow cytometer (model FACSCalibur, Becton Dickinson, USA) equipped with 488 nm Argon and 635 nm Helium–Neon lasers that emitted fluorescence (585/42 nm). In this experiment, cells with damaged cytoplasm membranes were coloured with PI. Viability was expressed as a percentage of uncoloured cells.

Meloxicam and control with DMSO were tested under the same experimental conditions. All assays were performed in triplicate.

2.3. Antiproliferative effect of meloxicam in tumoural cell lines

2.3.1. Cell lines

The cell lines employed were PC3 and DU-145, both human prostate cancer cell lines, PC3 cells have high metastatic potential when compared to DU-145 cells which have a moderate metastatic potential (Pulukuri et al., 2005; Alimirah et al., 2006). PC3 and DU-145 cell lines were obtained from American Type Culture Collection (ATCC, Manassaa, VA, USA). Cells were cultured under 5% CO_2 and 95% air atmosphere in a humidified incubator at 37 °C. Cells were incubated in a RPMI medium (Invitrogen, Spain) containing 10% fetal bovine serum (FBS), and 1 ml penicillin/streptomycin/fungizone (Invitrogen, Spain).

2.3.2. Antiproliferative assay

To test the effect of meloxicam on cell proliferation, PC3 and DU-145 cells (50×10^3 cells per well) were seeded in 24-well plates and allowed to adhere to the bottom of the wells for 24 h before the beginning of treatment. In an initial phase of the study, cells were exposed to increasing concentrations of meloxicam for 24 h (10–600 μM). Later, and after these results were obtained, PC3 cells were further exposed to the drug (concentration range 25–800 μM) for 24, 48 and 72 h. Meloxicam and control with DMSO were tested under the same experimental conditions. All assays were performed in triplicate.

At the end of the incubation periods, surviving cells were quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. This test measures the enzymatic activity of lactate dehydrogenase (LDH) in viable cells by means of the formation of an insoluble and coloured compound that can be quantified at 570/630 nm of optical density (OD). The antiproliferative effect of meloxicam was expressed as (OD of treated cells/OD of non-treated cells) × 100. IC_{50} (inhibition concentration 50%) was also calculated by plotting the log of percentage of inhibition values versus the drug concentrations assayed (Waskewich et al., 2002). Triplicate cultures in three independent experiments were performed.

2.4. Statistical analysis

Repeated measures analysis of variance was used to determine the overall significance of the effect of various concentrations of meloxicam or different incubation times on total cell count numbers. The level of statistical significance was set at 0.01.

2.5. Preparation of microspheres

Microspheres were prepared by the solvent evaporation technique from an o/w emulsion using two different amounts of PLGA (200 and 400 mg) and, with or without the addition of PEG-derivative. Briefly, the corresponding amount of PLGA was dissolved into 4 ml CH_2Cl_2 by vortex mixing. A fixed amount of

meloxicam (15 mg) was dissolved in the organic phase under agitation to obtain a drug:polymer ratio of 0.75:10 and 0.375:10, respectively. The external phase of the emulsion consisted of 1% aqueous solution of PVA (20 ml). The organic phase was emulsified in the external aqueous phase using a homogenizer (IKA, Eurostar, Germany) at 2000 rpm for 15 min. The immature microspheres were suspended in 15 ml of distilled water and the system was continuously stirred for 4 h at room temperature to allow complete evaporation of the organic solvent.

Microspheres containing PEG-derivative were obtained by incorporating the additive (20 or 40 μl) into the inner phase of the emulsion in order to maintain an oil:polymer ratio of 1:10. Finally, the microspheres were vacuum filtered using 5 μm filters and vacuum dried at 25 °C for 48 h. Similarly, non-loaded PLGA-microspheres with and without PEG-derivative were prepared. In all cases, each formulation was prepared in triplicate batches.

2.6. Microspheres morphology

Microspheres particle shape and particle surface morphologies were studied by scanning electron microscopy (SEM, JEOL, JSM 6400, Tokyo, Japan). Samples were coated with a thin layer of colloidal gold applied in a cathodic vacuum evaporator before observation by SEM at 20 kV. The mean diameter and size distribution of each microsphere batch were evaluated by laser diffraction using a Galai model Cis-1 computerized inspection system (Migdal Haemek, Israel) within the 0.5–150 μm range.

2.7. Drug entrapment efficiency and process yield

A weighed amount of microspheres (10 mg) was dissolved into 5 ml of acetonitrile FAR-UV. After dilution with the mobile phase all samples were filtered through 0.45 μm filters and the meloxicam content of the microspheres was quantified by HPLC (Agilent 1100, Nagold, Germany) at 355 nm. The HPLC method used a C16 chromatographic column (Ascentis RP Amide C16, 15 cm, particle size 3 μm) and a flow rate of 0.5 ml/min. The mobile phase consisted of acetonitrile:0.3% formic acid solution adjusted at pH 2.8 (40:60, v/v). The components of the microspheres did not interfere with meloxicam at this wavelength. All the analyses were performed at 25 ± 0.5 °C and each determination was made in triplicate.

Encapsulation efficiency (E.E., %) was calculated as the ratio between the amount of drug content in the microspheres and the amount of drug used for the preparation of the microspheres. Process yield was calculated as the ratio between the total weight of microspheres obtained and the total weight of drug, polymer and other non-volatile solids added (if applicable).

2.8. Powder X-ray diffraction analysis

X-ray diffraction patterns were obtained using a Philips X'Pert model MPD (Almedo, The Netherlands) with a Cu Kα radiation, θ–2θ powder diffractometer set for an angle range of 5–50°/2θ, step size of 0.04°2θ and count times of 1 s per step. Samples of meloxicam, PLGA, non-loaded PLGA-microspheres prepared with and without PEG-derivative and, meloxicam PLGA-microspheres prepared with and without PEG-derivative were all scanned using these settings.

2.9. Differential scanning calorimetry (DSC)

In order to determine the physical state of the drug (i.e. amorphous or crystalline) and to evaluate any possible drug–polymer and/or drug–other components interactions, DSC scans were obtained using a Mettler DSC TA8000 calorimeter (Greinfensee, Switzerland) with nitrogen as purge gas. Samples of 10 mg were placed into aluminium pans and sealed using an empty pan for reference. The temperature range tested was 20–290 °C with a heating rate of 10 °C/min. DSC thermograms were obtained for samples of 10 mg meloxicam, PLGA, non-loaded PLGA-microspheres prepared with and without PEG-additive and, meloxicam PLGA-microspheres prepared with and without PEG-additive.

2.10. In vitro release study

In vitro drug release assays were carried out in a heated bath (Memmert WB22, Germany) at 37 ± 0.2 °C and constant shaking at 50 rpm. Microspheres (20 mg) were suspended in 4 ml of phosphate buffer saline (PBS) at pH 7.4 (sink conditions). At regular time intervals and up to 35 days, all PBS volume was withdrawn, centrifuged, filtered through 0.45 μm filters and the drug was quantified by UV spectrophotometry at 362 nm. At each time interval 4 ml of fresh PBS is used to replace volume withdrawal. Results obtained by spectrophotometry were confirmed by HPLC. All assays were done in triplicate for each formulation.

3. Results and discussion

Prostate cancer is presently the second leading cause of cancer-related death and the most commonly diagnosed non-skin cancer in men. PCa deaths are a result of metastatic disease and treatment of such metastatic disease is one of the major therapeutic challenges. Thus, there is a need to develop novel therapeutic approaches and strategies for successful chemotherapy. Meloxicam was introduced as an anti-inflammatory drug before the discovery of COX-2 and in recent years, experimental, epidemiological, and clinical studies have identified COX-inhibitors as promising compounds in combination therapy with chemotherapeutic agents (Wolfesberger et al., 2006; Suzuki et al., 2009).

Meloxicam has proven to inhibit cell proliferation of different cancer cell cultures but this effect has not yet been investigated in prostate cancer cell lines; therefore, we have studied the antoproliferative effect of different concentrations of meloxicam on PC3 and DU-145 cell lines. Moreover, taking into consideration that previous studies have shown that the effect of meloxicam on cell viability was dependent on drug concentration and could be time-dependent (Wolfesberger et al., 2006), we have also studied the effect of meloxicam alone on prostate cancer cells (PC3 and DU-145) treated with increasing concentrations of the drug (10–800 μM) for 24, 48 and 72 h.

In order to be able to evaluate the effect of meloxicam in non-tumoural cells, the normal growth of mouse fibroblast 3T3 cell was evaluated after exposure for 48 h to a wide range of concentrations of meloxicam. Fig. 1 shows the cell viability percentages obtained in NIH 3T3 cell line after exposure to 10, 25, 50, 100, 200 and 400 μM

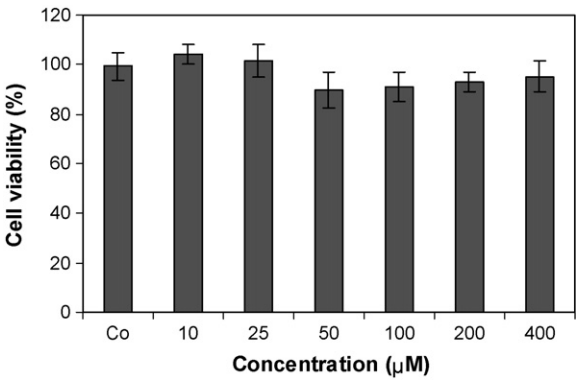

Fig. 1. Cell viability of NIH 3T3 cells after incubation for 48 h with different concentrations of meloxicam (μM). Co = control with DMSO. Bars represent mean ± SD.

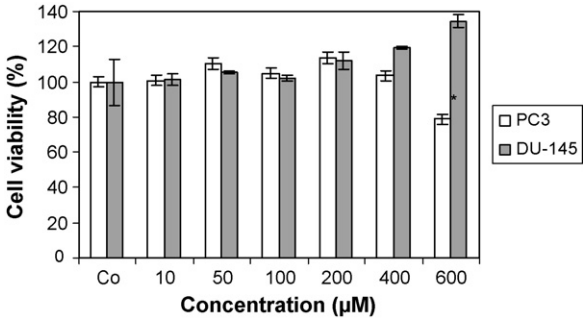

Fig. 2. Cell viability of PC3 cells and DU-145 cells after incubation for 24 h with different concentrations of meloxicam (μM). Bars represent mean ± SD. *Statistically significant differences from control group Co (with DMSO) ($p < 0.01$).

meloxicam. From the results obtained we have found no evidence of significant modification of cell viability after 48 h of cell exposure to the concentration range assayed.

Fig. 2 shows the antiproliferative results obtained after exposing PC3 and DU-145 human prostate cancer cell lines, respectively, to different concentrations of meloxicam (10–600 μM). Regarding PC3 and after 24 h of treatment with the drug, statistically significant reduction of cell viability was only observed after exposure to 600 μM meloxicam ($p < 0.01$). With respect to the results obtained for DU-145 cells, an increasing tendency in cell growth rate after exposure to 200, 400 and 600 μM meloxicam occurred however, this increase was only statistically significant at the highest concentration tested (600 μM) ($p < 0.01$).

These results reveal that meloxicam has an antiproliferative effect against PC3 but not against DU-145 prostate cancer cells which shows the ability of the drug to exert its selective antiproliferative effect on cell lines having high metastatic potential. It could be that the DU-145 prostate cancer line is less sensitive to COX-2 inhibitors than other PCa cell lines. We may also note that since even high concentrations of meloxicam did not inhibit the proliferation of DU-145 cells, high concentrations of the drug seem not to be cytotoxic in these cells. Our results are in agreement with the observation of Naruse et al. (2006) who studied the antiproliferative effect of meloxicam on several human and murine osteosarcoma cells indicating that this effect might differ significantly between cancer cell lines.

From these results and taking into consideration that the antiproliferative effect of meloxicam demonstrated in PC3 cells could also be time-dependent, further studies were performed after incubation of the drug at a wider concentration range (25–800 μM) for 24, 48 and 72 h (Fig. 3). After 24 h of treatment, a significant

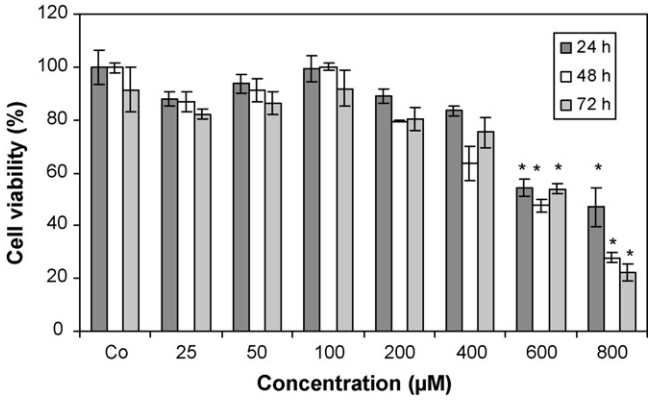

Fig. 3. Cell viability of PC3 cells after incubation for 24, 48 and 72 h with different concentrations of meloxicam (μM). Bars represent mean ± SD. *Statistically significant differences from control group Co (with DMSO) ($p < 0.01$).

reduction of cell viability was observed in the presence of 600 and 800 μM meloxicam ($p < 0.01$) when compared with control cells. A significant reduction in cell numbers was also noted after exposure to concentrations of 600 and 800 μM meloxicam for 48 and 72 h ($p < 0.01$). The IC_{50} decreased from 740 μM at 24 h to 499 μM at 48 h and 515 μM at 72 h after meloxicam treatment.

Our results confirm that the lack of cytotoxicity of meloxicam on NIH 3T3 cells when compared to PCa cell lines demonstrates the selective cytotoxic effect of meloxicam on PC3 cells and that this effect is both dose- and time-dependent; therefore, we should consider meloxicam as a possible adjuvant agent in anticancer therapy, bearing in mind that further in vitro and in vivo studies are needed. The clinical relevance of our results remains to be established, but data from prostate cancer cell lines exhibiting high metastatic potential have shown positive findings.

To date, no data concerning intratumoural concentrations of meloxicam are available however, in our work, as in other studies (Busch et al., 1998), the concentrations of meloxicam assayed were higher than the physiological maximum serum concentration (0.734 mg/l), which raises the question of whether or not the concentration in the tumour cells is within the same range as measured in the serum. In order to be able to achieve high enough effective intratumoural concentrations of meloxicam in prostate cancer we have developed a new controlled release system consisting of PLGA-microspheres. This new therapeutic system will allow maximizing the efficiency of the drug after direct administration near the tumour by means of chemoembolization by transcatheter arterial prostatic administration. In the last few decades embolization has been used extensively to occlude vessels as an alternative way of administration when traditional therapy failed (Robert et al., 2004). Transcatheter arterial embolization has many advantages, including good targeting, high therapeutic effects, and light trauma (Chen, 1998). Embolization and chemotherapy have been combined to treat malignant tumour, mostly incorporating chemotherapeutics perfused or injected into tumour. The new controlled release system that we have developed has been prepared with PLGA, a biodegradable polymer that offers the advantage of its biodegradation, being able to disappear gradually while releasing the drug from the site of action.

For the preparation of meloxicam microspheres PLGA Resomer® RG 503 (Mw = 34 kDa) was selected since in previous studies regarding the effect of polymer molecular weight on the encapsulation efficiency, this resomer showed very good results for encapsulating liphophilic molecules, such as ibuprofen and indomethacin (Fernández-Carballido et al., 2004; Puebla et al., 2005). These studies showed higher encapsulation efficiencies with low molecular weight PLGA which in turn also exhibited biodegradability.

Different formulations of microspheres were developed using a fixed amount of meloxicam (15 mg), 200 or 400 mg of PLGA and, with or without the addition of PEG-derivative (Table 1). In all cases, non-loaded PLGA-microspheres were also prepared. PEG-derivative (Labrafil® 1944CS) was used in order to modulate the release rate of meloxicam from the microspheres. This PEG-derivative has been successfully used by us in previous studies for the formulation of PLGA/PEG-derivative microspheres of indomethacin (Puebla et al., 2005).

Fig. 4 shows the SEM microphotographs of all formulations obtained for meloxicam PLGA-microspheres prepared according to Table 1. As it can be seen, microspheres prepared without the incorporation of PEG-derivative were spherical in shape with smooth surfaces whereas microspheres prepared with PEG-derivative showed concavities which were homogeneously distributed throughout the surface. Previous studies have indicated that this fact could be probably caused by the plasticity effect of PEG-derivative or by its rapid precipitation during the evaporation

Table 1
Formulations of meloxicam PLGA-microspheres prepared and results obtained for particle size (μm), encapsulation efficiency (E.E.,%), and amount of meloxicam (μg) encapsulated per mg of microspheres (±SD).

Formulation	Meloxicam (mg)	PLGA (mg)	Drug/polymer ratio	PEG-der./polymer ratio	Particle size (μm)	E.E. (%)	μg meloxicam/mg microspheres
A0	15	200	7.5%	–	9.34 ± 0.09	59.97 ± 8.22	41.84 ± 8.11
A1	15	200	7.5%	10%	13.63 ± 0.08	75.97 ± 1.36	53.00 ± 1.33
B0	15	400	3.75%	–	11.81 ± 0.15	63.80 ± 17.43	23.06 ± 6.30
B1	15	400	3.75%	10%	13.06 ± 0.09	58.44 ± 4.53	21.12 ± 1.63

phase in the microsphere preparation process (Hedberg et al., 2002; Fernández-Carballido et al., 2008). The same morphology changes were observed in non-loaded PLGA-microspheres (SEM microphotographs not shown).

Particle size is a key parameter when developing a new formulation for chemoembolization. In our case, particle sizes of all experimental meloxicam PLGA formulations prepared (Table 1) showed unimodal distributions with mean diameters ranging from 9.34 ± 0.09 to 13.63 ± 0.08 μm, being these sizes considered suitable for potential chemoembolization (Freitas, 1999). Mean particle size was significantly higher ($p < 0.05$) for the microspheres prepared with PEG-derivative (formulations A1 and B1) when compared with the microspheres prepared without this oil additive (formulations A0 and B0). When preparing the different microspheres formulations the amount of PLGA used was increased from 200 to 400 mg, however, in all cases mean particles sizes were smaller than 15 μm, which could be explained by the fact that the viscosity of the organic phase was low in all formulations for the same rotating speed (Freitas et al., 2005).

Process yields obtained were higher than 90% for all formulations prepared. Mean encapsulation efficiencies of meloxicam in the formulations prepared ranged from 58.44 ± 4.53% to 75.97 ± 1.36%, which correspond to drug loadings of 2.11 ± 0.16% and 5.30 ± 0.13%, respectively (Table 1). The highest encapsulation efficiency was obtained for formulation A1 corresponding to meloxicam PLGA-microspheres prepared with PEG-derivative which improves the affinity of the drug for the polymeric matrix. These results are in disagreement with those obtained by other authors (Freitas et al., 2005; Coimbra et al., 2008) who reported an increase of the encapsulation efficiency with increasing polymer concentration, explaining this fact by the viscosity increase of the inner phase, which restricts the drug migration to the continuous phase. In our case, and as indicated previously, the viscosity of the organic phase was low in all the microspheres formulations prepared.

The internal structure of the microspheres was studied by X-ray powder diffraction analysis. The diffraction pattern of meloxicam was characteristic of a crystalline state with maxima of intensity at 13.14°, 14.98°, 18.66°, 19.34°, 25.86°, and 29.66° (patterns not shown). The diffraction pattern obtained for PLGA showed no maximum, confirming its amorphous state. Non-loaded PLGA-microspheres with PEG-derivative presented a similar diffraction pattern to the one obtained for the polymer, which indicates that PEG-derivative and the encapsulation process did not affect the polymer characteristics. All meloxicam PLGA-microspheres prepared with or without PEG-derivative showed an irregular baseline without the strongest maxima of meloxicam, which suggests a loss of crystallinity of the drug inside the microspheres.

The DSC scan of meloxicam displayed a narrow endothermic peak at 255.86 °C, which corresponds to the solid melting point (*Tm*) of the drug. The thermogram of PLGA showed an endothermic peak at 45.85 °C, corresponding to its glass transition temperature (*Tg*). When scanning non-loaded PLGA-microspheres prepared without PEG-derivative, a similar *Tg* value was obtained (45.01 °C) however, the *Tg* value obtained for non-loaded PLGA-microspheres

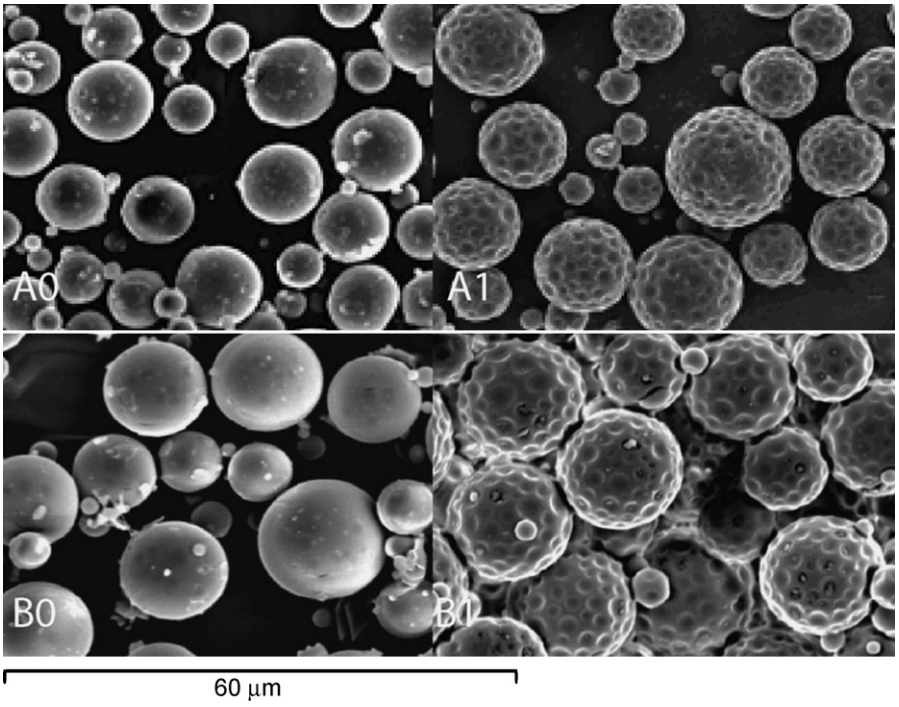

Fig. 4. Scanning electron microphotographs (1000×) of meloxicam PLGA-microspheres formulations A0, A1, B0, and B1 prepared according to Table 1.

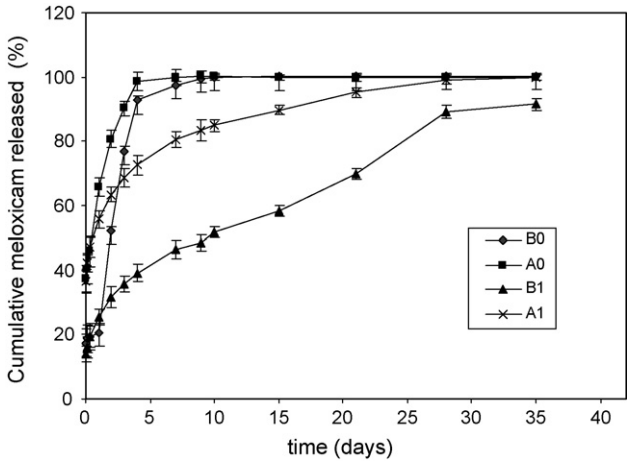

Fig. 5. Cumulative percentages of meloxicam released from PLGA-microspheres prepared without PEG-derivative (formulations A0 and B0) and, with PEG-derivative (formulations A1 and B1).

prepared with PEG-derivative was slightly lower (43.80 °C), probably due to a smoothness effect of the PEG-derivative. The thermal scans of meloxicam PLGA-microspheres prepared with or without PEG-derivative (formulations B0 and B1) showed the characteristic endothermic peak of the polymer but the endothermic melting peak of meloxicam was absent. Dubernet (1995) discussed the possible interactions between the drug and its polymer microsphere matrix, and their effects on the thermal properties of the microspheres. According to this author, if the drug is initially dissolved during the microsphere preparation procedure, as in our case, the disappearance of the drug endothermic peak in the DSC profile of the drug-loaded microspheres could indicate that the drug is dissolved in the polymer as a solid solution or that the drug is dispersed in the polymer matrix in a metastable molecular dispersion. These results are in agreement with our data obtained by X-ray powder diffraction analysis.

The in vitro release profiles obtained for meloxicam microspheres are shown in Fig. 5. Initial burst (1 h) was significantly higher ($p < 0.05$) for A0 and A1 microspheres which were prepared with the lowest amount of PLGA (200 mg) when compared to formulations B0 and B1 prepared with 400 mg of PLGA. For A0 and A1 microspheres, mean initial burst release was around 37% of the drug released within the first 1 h of the in vitro assay, whereas the initial burst obtained for formulations B0 and B1 was around 15% of the drug. From these results it can be stated that the addition of PEG-derivative did not have a significant influence on the initial burst release of meloxicam microspheres.

The release rate of meloxicam from the formulations which was prepared without PEG-derivative (formulations A0 and B0) was very fast, thereby resulting in the release of the total amount of meloxicam encapsulated (100% released) within the first 9 days of the in vitro release test. These types of release patterns are usually observed with small microparticles. For meloxicam PLGA/PEG-derivative microspheres (formulation B1), after 3 days of the in vitro assay around 35% of the drug is released. From that time point, a zero-order kinetics was maintained for 28 days with a release rate constant of 0.45 ± 0.05 μg meloxicam/day/mg microspheres. After 28 days more than 89% of the drug was released from the microspheres. In the case of meloxicam PLGA/PEG-derivative microspheres prepared with a lower polymer concentration (formulation A1), two consecutive release kinetics occurred; the first one resulted in a release of 68% of meloxicam after 3 days of the assay and, the second one, a very slow release which exhibited a zero-order release rate of 0.16 ± 0.03 μg meloxicam/day/mg microspheres. During this zero-order kinetics the amount of drug released would be insufficient to achieve effective antiproliferative levels. After 35 days, formulation A1 released 100% of the drug encapsulated.

These results indicate that the incorporation of PEG-derivative to the microspheres significantly affected the release rate of meloxicam from PLGA-microspheres. Previous studies have also shown that PEG-derivative retards the release rate of other anti-inflammatory agents, such as indomethacin, from PLGA-microspheres due to a loss of porosity of the polymeric matrix when the additive is present (Fernández-Carballido et al., 2008). Moreover, PEG-derivative could also modify the diffusion coefficient of the drug (Lemaire et al., 2003).

For selecting the more suitable formulation it is important to obtain effective drug release according to a zero-order kinetics during an extended period of time. In vitro drug release assays indicated that microspheres prepared without PEG-derivative (formulations A0 and B0) released meloxicam at a rate excessively fast. Of all the formulations studied, only formulation B1 (prepared with meloxicam:polymer ratio of 3.75% and PEG-derivative:polymer ratio of 10%) presented a zero-order release kinetics between days 3 and 28 which will be appropriate to achieve effective in vivo antiproliferative levels. This fact together with the small particles sizes obtained for the microparticles make this new formulation suitable for chemoembolization.

4. Conclusions

In conclusion this study provides evidence of a selective antitumour effect of meloxicam on the human prostate cancer cell line, PC3, in vitro which was also dose- and time-dependent. The new formulation developed consisting of biodegradable meloxicam PLGA/PEG-derivative microspheres is adequate for chemoembolization and will release the drug according to a zero-order kinetics for approximately 4 weeks prolonging the tumoural concentration of the drug and when used in combination with chemotherapy, meloxicam may prove to have a therapeutic role in counteracting the tumourigenicity of PCa. Further studies of its clinical use in PCa are required.

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References

Alimirah, F., Chen, J., Basrawala, Z., Xin, H., Choubey, D., 2006. DU-145 and PC-3 human prostate cancer cell lines express androgen receptor: implications for the androgen receptor functions and regulation. FEBS Lett. 580, 2294–2300.

Bendszus, M., Klein, R., Burger, R., Warmuth, M.M., Hofmann, E., Solymosi, L., 2000. Efficacy of trisacryl gelatin microspheres versus polyvinyl alcohol particles in the preoperative embolization of meningiomas. Am. J. Neuroradiol. 21, 255–261.

Busch, U., Schmid, J., Heinzel, G., Schmaus, H., Baierl, J., Huber, C., Roth, W., 1998. Pharmacokinetics of meloxicam in animals and the relevance to humans. Drug Metab. Dispos. 26, 576–584.

Chen, C.X., 1998. Practical Radiology. People's Health Press, China, Beijing, pp. 1058–1060.

Coimbra, P.A., De Sousa, H.C., Gil, M.H., 2008. Preparation and characterization of flurbiprofen-loaded poly(3-hydroxybutyrate-co-3-hydroxyvalerate) microspheres. J. Microencapsul. 25, 170–178.

Dubernet, C., 1995. Thermoanalysis of microspheres. Thermochim. Acta 248, 259–269.

Falandry, C., Canney, P.A., Freyer, G., Dirix, L.Y., 2009. Role of combination therapy with aromatase and cyclooxygenase-2 inhibitors in patients with metastatic breast cancer. Ann. Oncol. 20, 615–620.

Fernández-Carballido, A., Herrero-Vanrell, R., Molina-Martínez, I.T., Pastoriza, P., 2004. Biodegradable ibuprofen-loaded PLGA microspheres for intraarticular

administration. Effect of labrafil addition on release in vitro. Int. J. Pharm. 279, 33–41.
Fernández-Carballido, A., Pastoriza, P., Barcia, E., Montejo, C., Negro, S., 2008. PLGA/PEG-derivative polymeric matriz for drug delivery system applications: characterization and cell viability studies. Int. J. Pharm. 352, 50–57.
Fosslien, E., 2000. Biochemistry of cyclooxygenase (COX)-2 inhibitors and molecular pathology of COX-2 in neoplasia. Crit. Rev. Clin. Lab. Sci. 37, 431–502.
Freitas Jr., R.A., 1999. Nanomedicine, Volume I: Basic Capabilities. Landes Bioscience, Georgetown, TX.
Freitas, S., Merkle, H.P., Gander, B., 2005. Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology. J. Control. Release 102, 313–332.
Gao, Z.H., Crowley, W.R., Shukla, A.J., Johnson, J.R., Reger, J.F., 1995. Controlled release of contraceptive steroids from biodegradable and injectable gel formulations: *in vivo* evaluation. Pharm. Res. 12, 864–868.
Gasparini, G., Longo, R., Sarmiento, R., Morabito, A., 2003. Inhibitors of cyclooxygenase 2: a new class of anticancer agents? Lancet Oncol. 4, 605–615.
Goldman, A.P., Williams, C.S., Sheng, H., Lamps, L.W., Williams, V.P., Pairet, M., Marrow, J.D., DuBois, R.N., 1998. Meloxicam inhibits the growth of colorectal cancer cell. Carcinogenesis 19, 2195–2199.
Graff, J.R., Deddens, J.A., Konicek, B.W., Colligan, B.M., Hurst, B.M., Carter, H.W., Carter, J.H., 2001. Integrin-linked kinase expression increases with prostate tumor grade. Clin. Cancer Res. 7, 1987–1991.
Gupta, S., Srivastava, M., Ahmad, N., Bostwick, D.G., Mukhtar, H., 2000. Overexpression of cyclooxygenase-2 in human prostate adenocarcinoma. Prostate 42, 73–78.
Han, Z.D., Bi, X.C., Qin, W.J., He, H.C., Dai, Q.S., Zou, J., Ye, Y.K., Liang, Y.X., Zeng, G.H., Chen, Z.N., Zhong, W.D., 2008a. CD147 expression indicates unfavourable prognosis in prostate cancer. Pathol. Oncol. Res., doi:10.1007/s12253-008-9131-z.
Han, Z.D., He, H.C., Bi, X.C., Qin, W.J., Dai, Q.S., Zou, J., Ye, Y.K., Liang, Y.X., Zeng, G.H., Zhu, G., Chen, Z.N., Zhong, W.D., 2008b. Expression and clinical significance of CD147 in genitourinary carcinomas. J. Surg. Res. 10, 1–8.
Hedberg, E.L., Tang, A., Crowther, R.S., Carney, D.H., Mikos, A., 2002. Controlled release of an osteogenic peptide from injectable biodegradable polymeric composites. J. Control. Release 84, 137–150.
Kern, M.A., Schoneweis, M.M., Sahi, D., Bahlo, M., Haugg, A.M., Kasper, H.U., Dienes, H.P., Kaferstein, H., Breuhahn, K., Schirmacher, P., 2004. Cyclooxygenase-2 inhibitors suppress the growth of human hepatocellular carcinoma implants in nude mice. Carcinogenesis 25, 1193–1199.
Lemaire, V., Belair, J., Hildgen, P., 2003. Structural modelling of drug release from biodegradable porous matrices based on a combined diffusion/erosion process. Int. J. Pharm. 258, 95–107.
Liao, J., Schneider, A., Datta, N.S., McCauley, L.K., 2006. Extracellular calcium as a candidate mediator of prostate cancer skeletal metastasis. Cancer Res. 66, 9065–9073.
Naruse, T., Nishida, Y., Hosono, K., Ishiguro, N., 2006. Meloxicam inhibits osteosarcoma growth, invasiveness and metastasis by COX-2-dependent and independent routes. Carcinogenesis 27, 584–592.
Nelson, W.G., De Marzo, A.M., Isaacs, W.B., 2003. Prostate cancer. N. Engl. J. Med. 349, 366–381.
Puebla, P., Pastoriza, P., Barcia, E., Fernández-Carballido, A., 2005. PEG-derivative effectively modifies the characteristics of indomethacin-PLGA microspheres destined to intra-articular administration. J. Microencapsul. 22, 793–808.
Pulukuri, S.M., Gondi, C.S., Lakka, S.S., Jutla, A., Estes, N., Gujeari, M., Rao, J.S., 2005. RNA interference-directed knockdown of urokinase plasminogen activator and urokinase plasminogen activator receptor inhibits prostate cancer cell invasion, survival, and tumorigenicity in vivo. J. Biol. Chem. 280, 36529–36540.
Rafael, M.N., Jacob, B., Marcelo, D., Micha, P., Dov, L., 2002. Selective embolization for head and neck—vasculopathies. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 93, 127–131.
Robert, A., Lookstein, M.D., Jeffrey, M.D., 2004. Embolization of complex vascular lesions. Mt. Sinal J. Med. 71, 17–28.
Suzuki, R., Yamamoto, M., Saka, H., Taniguchi, H., Shindoh, J., Tanikawa, Y., Nomura, F., Gouda, H., Imaizumi, K., Hasegawa, Y., Shimokata, K., 2009. A phase II study of carboplatin and paclitacel with meloxicam. Lung Cancer 63, 72–76.
Tsubouchi, Y., Mukai, S., Kawahito, Y., Yamada, R., Kohno, M., Inoue, K., Sano, H., 2000. Meloxicam inhibits the growth of colorectal cancer cells. Carcinogenesis 19, 2195–2199.
Tsuchida, A., Itoi, T., Kasuya, K., Endo, M., Katsumata, K., Auki, T., Suzuki, M., Aoki, K., 2005. Inhibitory effect of meloxicam, a cyclooxigenase-2 inhibitor, on N-nitrosobis (2-oxopropyl) amine induced biliary carcinogenesis in syrian hamsters. Carcinogenesis 26, 1922–1928.
Wang, J., Cai, Y., Penland, R., Chauhan, S., Miesfeld, R.L., Ittmann, M., 2006. Increased expression of the metastasis-associated gene Ehm2 in prostate cancer. Prostate 66, 1641–1652.
Waskewich, C., Blumenthal, R.D., Li, H., Stein, R., Goldenberg, D.M., Burton, J., 2002. Celocoxib exhibitors for growth inhibition of COX-2 negative hematopoietic and epithelial cell lines. Cancer Res. 62, 2029–2033.
Wolfesberger, B., Walter, I., Hoelzl, C., Thalhammer, J.G., Egerbacher, M., 2006. Antineoplastic effect of the cyclooxygenase inhibitor meloxicam on canine osteosarcoma cells. Res. Vet. Sci. 80, 308–316.
Yamamoto, T., Hayakawa, K., Tabata, Y., Shimizu, Y., Ikada, Y., 2003. Transcatheter arterial embolization using poly-L-lactic acid microspheres. Radiat. Med. 21, 150–154.