The efficacy of methotrexate-impregnated hydroxyapatite composites on human mammary carcinoma cells

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ABSTRACT

Purpose. To investigate the efficacy of local bio-degradable composites of hydroxyapatite, plaster of Paris, and a binder of either alginate or chitosan impregnated with methotrexate on human mammary carcinoma cells.

Methods. An *in vitro* analysis of drug dissolution and a cytotoxicity test on human mammary carcinoma cells were performed over one month. Physicochemical properties of each composite were investigated using scanning electron microscopy, X-ray diffractometry, and Fourier transform infrared spectroscopy.

Results. Both composites with a binder of either alginate or chitosan could release methotrexate for over one month. The amount of methotrexate released depended on the amount of methotrexate loaded. The composite using alginate as a binder released a significantly greater amount of methotrexate than that using chitosan as a binder (p<0.05). The elution of both composites showed favourable cytotoxicity when the concentration was greater than 5 µg/ml.

Conclusion. Methotrexate-impregnated hydroxyapatite composites appear to be effective local skeletal methotrexate delivery systems against human mammary carcinoma cells in an *in vitro* model.

Key words: breast neoplasms; hydroxyapatite cement; methotrexate

INTRODUCTION

Chemotherapy is a standard treatment for most malignant bone tumours. To achieve optimal effect, a high systemic concentration of drug for a sufficiently long period is required to achieve both local and systemic control. However, parenteral administration of chemotherapy drugs in high dose is associated with adverse effects such as immune suppression, myelosuppression, hepatotoxicity, and cardiotoxicity.1,2 Methods have been proposed to reduce such effects associated with systemic delivery of anticancer agents. Development of local drug delivery systems has been one approach to reduce the risk of systemic complications.3 Various drug
carriers such as polymers, hydroxyapatite, hydrogel, and poly(methylmethacrylate) have been studied with conflicting results.\textsuperscript{4-10} We have reported on the efficacy of a new composite of hydroxyapatite, plaster of Paris, and a binder of either chitosan or alginate for antibiotic and antifungal drugs.\textsuperscript{11-13} In the present study, we aimed to test the efficacy of these hydroxyapatite composites impregnated with methotrexate on human mammary carcinoma cells in an \textit{in vitro} model.

MATERIALS AND METHODS

Hydroxyapatite (CA\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}(OH)\textsubscript{2}) from MTEC, Bangkok, Thailand; plaster of Paris from Merck, Darmstadt, Germany; and a binder of either alginate or chitosan (medium viscosity, 80% acetylation) from Fluka, Buchs, Switzerland were used to make the hydroxyapatite composite, which was impregnated with methotrexate from Zetate 50, Dabur, India. A geometric dilution technique was used: 4.25 g of hydroxyapatite was well mixed with plaster of Paris at a ratio of 85:15. Chitosan and alginate gel was prepared by dispersing in distilled water at 1% concentration. The plaster of Paris and hydroxyapatite were thoroughly mixed before the methotrexate solution was added and the mixture stirred until it became a homogeneous and smooth paste. It was then poured into a silastic mould (6.5 mm in diameter and 5.2 mm in thickness). Three different concentrations of methotrexate—0.5%, 1%, and 2%—were impregnated in each of the 2 composites. One composite without methotrexate served as a control. After the cement tablets hardened, they were dried at 60°C for 2 hours and sterilised with ethylene oxide. Standard safety guidelines for using anticancer drugs were followed.

Five tablets of each preparation were tested for drug dissolution using a dissolution apparatus at 37°C with a stirring speed of 50 revolutions per minute. Each reactor contained an implant and 100 ml of phosphate buffer at pH 7.4. Three reactors were used for each preparation. Two samples were taken at different time points and filtered with a millipore filter and then kept at -25°C. Dissolution was tested over one month. The amount of methotrexate in the sample was determined by ultraviolet absorbance at 304 nm with a spectrophotometer.

Human mammary carcinoma cells were grown as a monolayer in EMEM (Eagle Minimum Essential Medium, Biochrom KG Seromed, Berlin, Germany) with 10% newborn calf serum (Biowhittaker, Walkersville [MD], US) and penicillin/streptomycin/amphotericin B (100 µg/ml). The cells were typically grown to 70 to 80% confluence under 5% CO\textsubscript{2} at 37°C in a CO\textsubscript{2} incubator. Then the medium was changed and the cells used for the test procedure. The cells were removed from the culture flasks by washing with phosphate-buffered saline (Biochrom KG, Berlin, Germany) and trypsinised, and then incubated for 3 minutes at 37°C in a CO\textsubscript{2} incubator and diluted with fresh medium. A total of 100 µl containing 3x10\textsuperscript{4} cells was then added to a 96-well plate, which was then incubated for 24 hours in a CO\textsubscript{2} incubator. Test samples were initially dissolved in dimethyl sulphoxide, then diluted into 10-fold serial dilutions with medium. Serial dilutions were performed and 100 µl was added to each well. After 72 hours of incubation, the cells were washed with fresh medium, cultured for an additional 72 hours, and then fixed with 100 µl of cold 40% aqueous trichloroacetic acid. The plates were kept at 4°C for one hour, washed with tap water 5 times and air-dried. The cells were stained using 0.4% sulphorhodamine B in 1% acetic acid (50 µl) for 30 minutes. Excess sulphorhodamine B solution was removed by washing 5 times with 1% aqueous acetic acid. Once dried, the bound dye was dissolved in 100 µl 10 mM Tris base pH10. The plate was shaken for 5 minutes, and the absorbance was measured at 492 nm using a microplate reader (Power Wave X, Bio-Tex Instruments, Winooski [VT], US). Finally, the results were reported in terms of median effective dose, calculated using toxicity percentage versus concentration. An extract was considered active when the median effective dose was <20 µg/ml.

Three samples of each preparation were examined by scanning electron microscopy, X-ray diffractometry, and Fourier transform infrared spectroscopy. For the scanning electron microscopy, each carrier was pasted onto a brass stub and coated with a 20-mm gold layer. (JEOL, Model SM-5800 LV, Tokyo, Japan). For X-ray diffractometry, the sample was prepared by powdered compression technique. The diffraction patterns were obtained from a diffractometer (Philips Xpert MPD, Amelo, Netherlands), at an angle of 50° to 90°.

Means and standard deviations of methotrexate concentrations were calculated for each preparation daily. To evaluate the statistical significance of differences in methotrexate concentration between carriers, the generalised estimating equations method of linear regression was used on each day of testing.

RESULTS

Methotrexate eluted from either composite followed
a similar pattern. At the initial phase, all composites quickly released methotrexate and then the elution plateaued after one week. The release profiles depended on the amount of methotrexate loaded. The higher the concentration of methotrexate loaded, the greater the amount eluted. Both hydroxyapatite composites using a binder of either alginate or chitosan released methotrexate for at least one month. However, at the same time point at each concentration, the hydroxyapatite composites using alginate as a binder showed a significantly greater amount of methotrexate elution than that using chitosan as a binder (Table).

Eluted methotrexate had a cytotoxic effect on human mammary carcinoma cells depending on the amount of methotrexate loaded. At a concentration of 5 µg/ml, >80% of the tumour cells were killed. The eluted methotrexate from both composites demonstrated a significantly greater cytotoxic effect on human mammary carcinoma cells at a concentration of 5 µg/ml than at ≤5 µg/ml. Concentrations of >5 µg/ml showed little increase in cytotoxic activity. There was no significant difference between cytotoxic effects of the 2 composites at the same concentration of methotrexate elution than that using chitosan as a binder (Table).

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Figure 1 Relationship of cytotoxicity and eluted methotrexate.

Figure 2 Histological changes of cells (a) before and (b) after cytotoxicity test.
DISCUSSION

This new biodegradable carrier composed of hydroxyapatite, plaster of Paris, and a binder of either chitosan or alginate is suitable for use as a local carrier of methotrexate directed against human mammary carcinoma cells in an in vitro model. Both composites provided a sustained release throughout a one-month period and had cytotoxic effects. The amount of methotrexate eluted depended on the amount of methotrexate loaded, as well as the elution time and the type of carrier. Our results indicated that the concentration of eluted methotrexate should be >5 µg/ml to have a cytotoxic effect of >80%. The kinetics of methotrexate release showed a similar pattern of release to that encountered with most antibiotic-impregnated local drug carrier systems.14

The initially high drug elution during the first week is due to the mechanism of drug release (mostly from the surface). The sustained release property of the 2 composites was better than that associated with using calcium phosphate,14 because both alginate and chitosan partially interact with calcium in both hydroxyapatite and plaster of Paris and also interact with the methotrexate, resulting in a slow release.15,16 Despite these interactions, the eluted methotrexate is still in an active form, as demonstrated by the cytotoxicity test.17 At every corresponding concentration, the hydroxyapatite composite using alginate as a binder showed a significantly better release profile than that using chitosan. However, there was no significant difference in cytotoxic effect against the human mammary carcinoma cells. This might be explained by the dose-response curve—the methotrexate concentration being above the threshold (minimum inhibitory/cytotoxic concentration) for the cancer cells.

Local skeletal drug delivery systems have been increasingly popular, because they offer advantages over parenteral treatment: fewer systemic side-effects, decreased hospital stays, and favourable efficacy.18–21 Most studies of local drug treatment focus mainly on antibiotics.3,5,12,14,19,21–24 Carrier systems using biodegradable materials have advantages over non-biodegradable carriers e.g. polymethylmethacrylate, because they do not need surgical removal, the risk of bacterial colonisation is decreased, and there is no thermal effect during polymerisation.5,13,20,23,25–27 In addition, hydroxyapatite, plaster of Paris, alginate, and chitosan are biocompatible and have osteoconductive properties,6,8,13,23,25,28 which might decrease risks associated with bone grafting. Furthermore, both alginate and chitosan display an inherent antitumour activity by stimulating the production of the
tumour-related factors interleukin-1 and interleukin-6 from human monocytes. Nonetheless, in our study the control composites (without methotrexate) demonstrated no cytotoxic activity.

Methotrexate—a folic acid anti-metabolite—has been widely used for the treatment of various malignant diseases such as acute leukaemia, non-Hodgkin’s lymphoma, choriocarcinoma, osteosarcoma, and breast cancer. Methotrexate disturbs haematopoietic, hepatic, pulmonary, kidney, and skeletal tissues. Many attempts have been made to decrease its side-effects and improve both its specificity and efficacy by means of conjugating it to a carrier, e.g. antibody, polysaccharide, or polymethylmethacrylate. In this study, a new local carrier system of hydroxyapatite composite was used to carry the methotrexate, thus improving the locally available concentration.

Metastatic bone tumours are still a challenging orthopaedic problem. Multi-drug treatment is usually required to improve both survival and quality of life. Localised control of metastasis depends on many factors such as location and type of tumour, extent of disease and condition of the patient. Although surgery is the most common treatment for most primary malignant bone tumours, it plays a minor role in the management of bone metastases, except for certain indications such as impending or pathologic fractures. Radiation, another modality commonly used, is an adjuvant treatment primarily used for pain control. Other treatments such as bisphosphonates have been studied, especially in breast cancer. Local chemotherapy may be another option for local control of malignant bone tumours. Caution must be exercised when extrapolating results of in vitro studies to the clinical setting. Methotrexate-impregnated hydroxyapatite composites appear to offer an effective local skeletal delivery system against human mammary carcinoma cells in our in vitro model.

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REFERENCES