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Evaluation of a New Hydroxyapatite Nanoparticle as a Drug Delivery System to Oral Squamous Cell Carcinoma Cells

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Abstract. *Background/Aim: Due to its abilities of substance adsorption and intracellular transportation, hydroxyapatite is a potential carrier in drug delivery systems (DDS). This in vitro study investigated whether newly-developed, highly-dispersive calcined hydroxyapatite nanoparticles with an average grain diameter of 20 nm (nano-SHAP) were suitable as a DDS for the drugs zoledronic acid (ZA), cisplatin, and carboplatin. Material and Methods: The effects of drug-bearing nano-SHAP on cell proliferation were assessed using three human oral squamous cell carcinoma cell lines (HSC-4, KOSC, and SAS) and one human breast cancer cell line (MCF-7). Results: Nano-SHAP alone did not affect proliferation of any cell line until a concentration of 1 μg/ml was reached. Although the effective concentration of ZA in ZA-bearing nano-SHAP differed, it inhibited cell proliferation better than ZA alone. Cisplatin and carboplatin-bearing nano-SHAP had the same effect as these drugs alone. Conclusion: The nano-SHAP system is of potential use as a drug delivery system.*

Hydroxyapatite (HAP), which has a structural formula of $Ca_{10}(PO_4)_6(OH)_2$, is the principal inorganic constituent of human bones and teeth (1, 2). HAP is widely used in medical applications, including treating bone defects, tissue engineering systems, and bioactive coatings on metallic osseous implants (1-3). Hydroxyapatite nanoparticle (nano-HAP) structures have a large surface area, high loading capacity, and are capable of intracellular transportation. As a result, nano-HAP has been explored as a therapeutic molecule for drug delivery systems (DDS) (2, 3). Since nano-HAP has different properties depending on morphology, size, and surface properties, there is currently no consensus on what nano-HAP characteristics are most suitable for DDS (2, 3). Therefore, nano-HAP with various structures are produced using various techniques (2, 3).

*Bis*phosphonates (BPs) are first-line drugs for osteolytic diseases, including osteoporosis, Paget's disease, and cancer-induced hypercalcemia (4). BPs act as potent osteoclastic bone resorption inhibitors by inhibiting osteoclast activity and inducing their apoptosis (4-6). Zoledronic acid (ZA) is a third-generation, nitrogen-containing BP and is potentially a greater inhibitor of bone resorption (4, 7). ZA is widely used to prevent metastatic cancer-induced bone diseases (4, 7). Moreover, the direct antitumor activity of ZA has been reported in many cancer cell types *in vitro*, including oral squamous cell carcinoma (OSCC) cells, demonstrating significant growth inhibition and apoptosis induction (4, 7-9). However, inhibition of cancer cell proliferation in clinical research using ZA is difficult because plasma concentrations rapidly peak after intravenous administration of the standard dose of ZA (4 mg) and decline to 1% of the peak within 24 h (4, 8, 10). For ZA to directly inhibit tumor cell proliferation, it is necessary to develop a new method of administration.

This study investigated whether newly developed, highly-dispersive calcined HAP nanoparticles with an average grain diameter of 20 nm (nano-SHAP) can be applied as a DDS of ZA or two antitumor drugs (cisplatin and carboplatin) using three human OSCC cell lines and one breast cancer cell line.

Materials and Methods

Cells and reagents. Three human oral squamous cell lines (HSC-4, KOSC, and SAS) and one human breast cancer cell line (MCF-7) were kindly provided by Dr. M Noguchi, Department of Oral and Maxillofacial Surgery, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama. The cells were cultured and maintained at 37°C and 5% CO_2 in a humidified atmosphere in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% fetal bovine serum (FBS; Biowest, Paris, France), 100 U/ml penicillin, and 100 μg/ml

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Key Words: Hydroxyapatite nanoparticle, drug delivery system, bisphosphonate, oral squamous cell carcinoma.

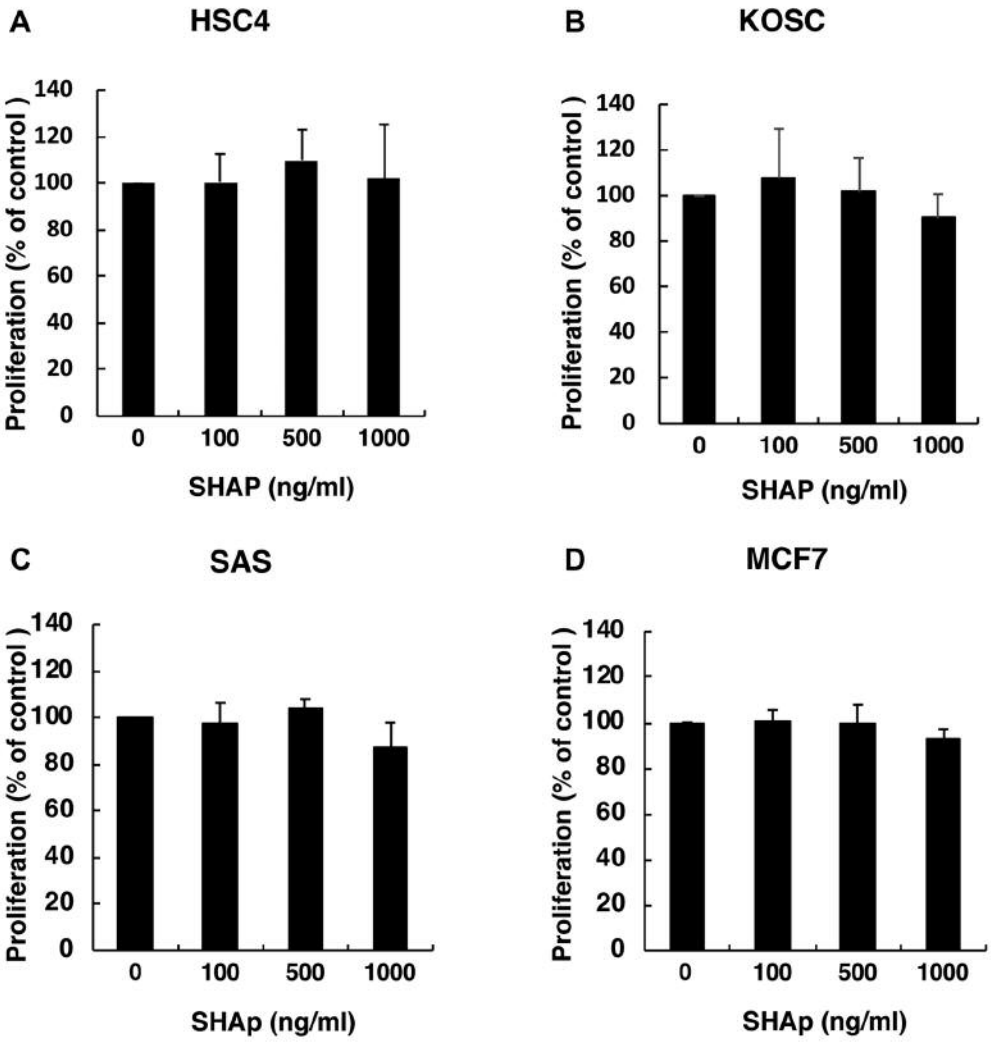

Figure 1. *Effect of highly-dispersive calcined hydroxyapatite nanoparticles (nano-SHAP) on cell proliferation of human oral squamous cell carcinoma cell lines HSC4 (A), KOSC (B), SAS (C) and human breast cancer cell line MCF-7 (D). The cells were exposed to increasing concentrations of nano-SHAP (100-1,000 ng/ml) for 72 h, and the effects on cell proliferation were measured using the MTS assay. All experiments were performed independently three times.*

streptomycin (Thermo Fisher Scientific). Highly-dispersive nano-SHAP with an average grain diameter of 20 nm were purchased from SofSera (Tokyo, Japan). ZA (Zometa®) was obtained from Novartis Pharmaceuticals (Basel, Switzerland). Cisplatin and carboplatin were purchased from Tocris Bioscience (Bristol, UK).

Preparation of drug-bearing nano-SHAP and nano-SHAP alone. Nano-SHAP was dissolved in distilled water, after which each drug was added and suspended. The mixture was kept at room temperature for 60 min. Nano-SHAP alone was prepared as follows. Nano-SHAP was dissolved in distilled water, and kept at room temperature for 60 min.

Cell proliferation assay. Cell proliferation was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA). In brief, the cells were plated at 2,000 cells per well in 96-well plates in RPMI 1640 medium containing 5% FBS; 24 h after plating, the medium was replaced with the same medium containing nano-SHAP alone, ZA-bearing nano-SHAP, cisplatin-bearing nano-SHAP or carboplatin-bearing nano-SHAP. After 72 h, the cell number was determined using the assay kit according to the manufacturer's instructions. Each sample's absorbance was measured at 490 nm using a microplate reader (Promega Corporation), which was then used to determine the relative cell count in each well. All experiments were performed independently three times.

Statistical analysis. Statistical analysis was performed using Student's unpaired *t*-test or one-way analysis of variance followed by Tukey's multiple comparison test. Statistical significance was defined as a calculated *p*-value of less than 0.05.

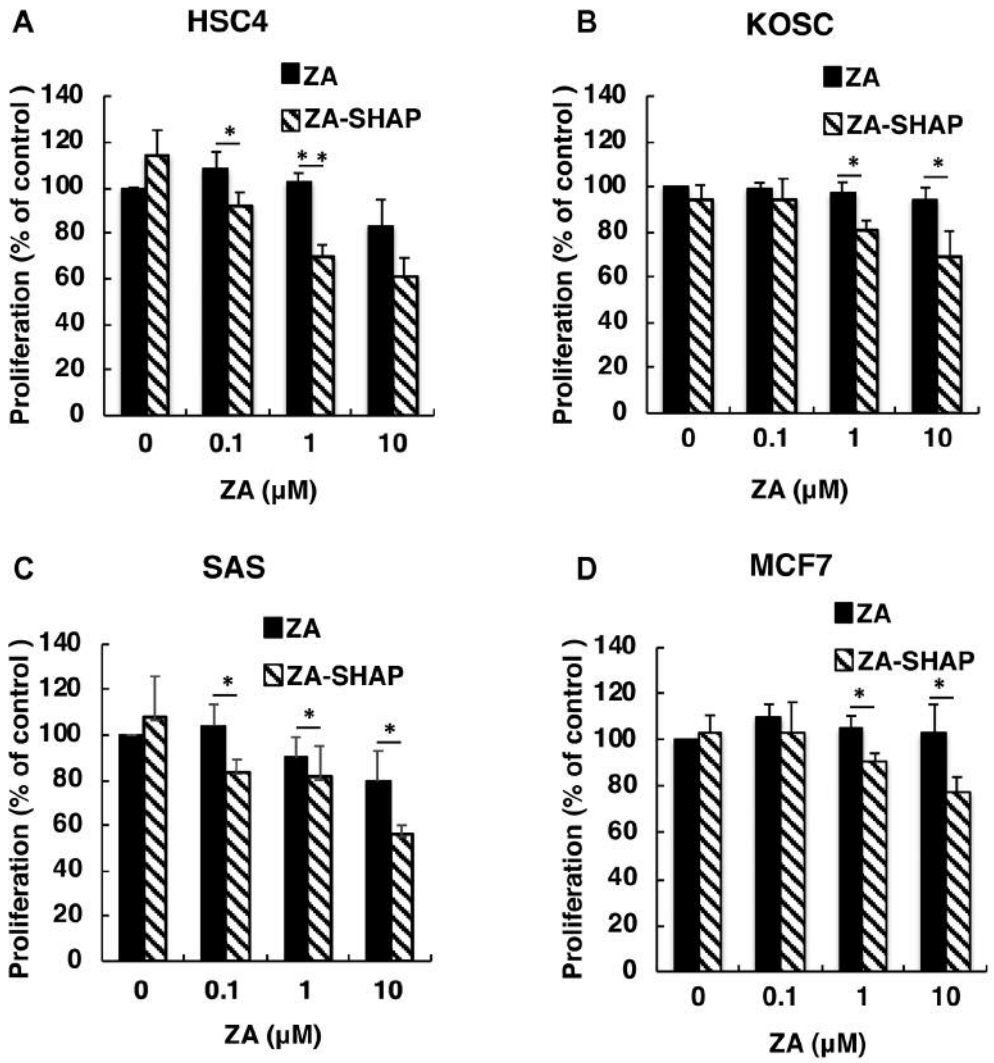

Figure 2. *Effect of zoledronic acid (Zometa®)-bearing highly-dispersive calcined hydroxyapatite nanoparticles (nano-SHAP) on cell proliferation of human oral squamous cell carcinoma cell lines HSC4 (A), KOSC (B), SAS (C) and human breast cancer cell line MCF-7 (D). Cells were exposed to increasing concentrations of ZA or ZA-bearing nano-SHAP (1,000 ng/ml) for 72 h, and the effects on cell proliferation were measured using the MTS assay. All experiments were performed independently three times. Significantly different at *p<0.05 and **p<0.01 versus the respective value without ZA.*

Results

Effect of nano-SHAP on cell proliferation. As the effect of nano-SHAP on cell proliferation in human OSCC and breast cancer cells was unclear, the effects of different concentrations of nano-SHAP on cell proliferation were assessed by the MTS assay. Nano-SHAP alone did not inhibit proliferation of any of the four cell lines at final concentrations ≤1,000 ng/ml (Figure 1).

Effect of ZA-bearing nano-SHAP on cell proliferation. Since 1,000 ng/ml nano-SHAP had no significant effect on cell proliferation, this concentration was used to examine the effect of ZA-bearing nano-SHAP. In HSC4 cells, ZA-bearing nano-SHAP significantly inhibited cell proliferation at 0.1 μM and 1 μM relative to ZA alone. In SAS cells, ZA-bearing nano-SHAP at >0.1 μM significantly inhibited cell proliferation. In KOSC and MCF-7 cells, ZA-bearing nano-SHAP at >1 μM significantly inhibited cell proliferation (Figure 2).

Effect of cisplatin-bearing nano-SHAP on cell proliferation. Cisplatin is a platinum-containing antitumor agent used to treat a variety of tumors, including SCC and breast cancer. The effect of cisplatin-bearing nano-SHAP on cell proliferation was examined. However, no differences were found between the

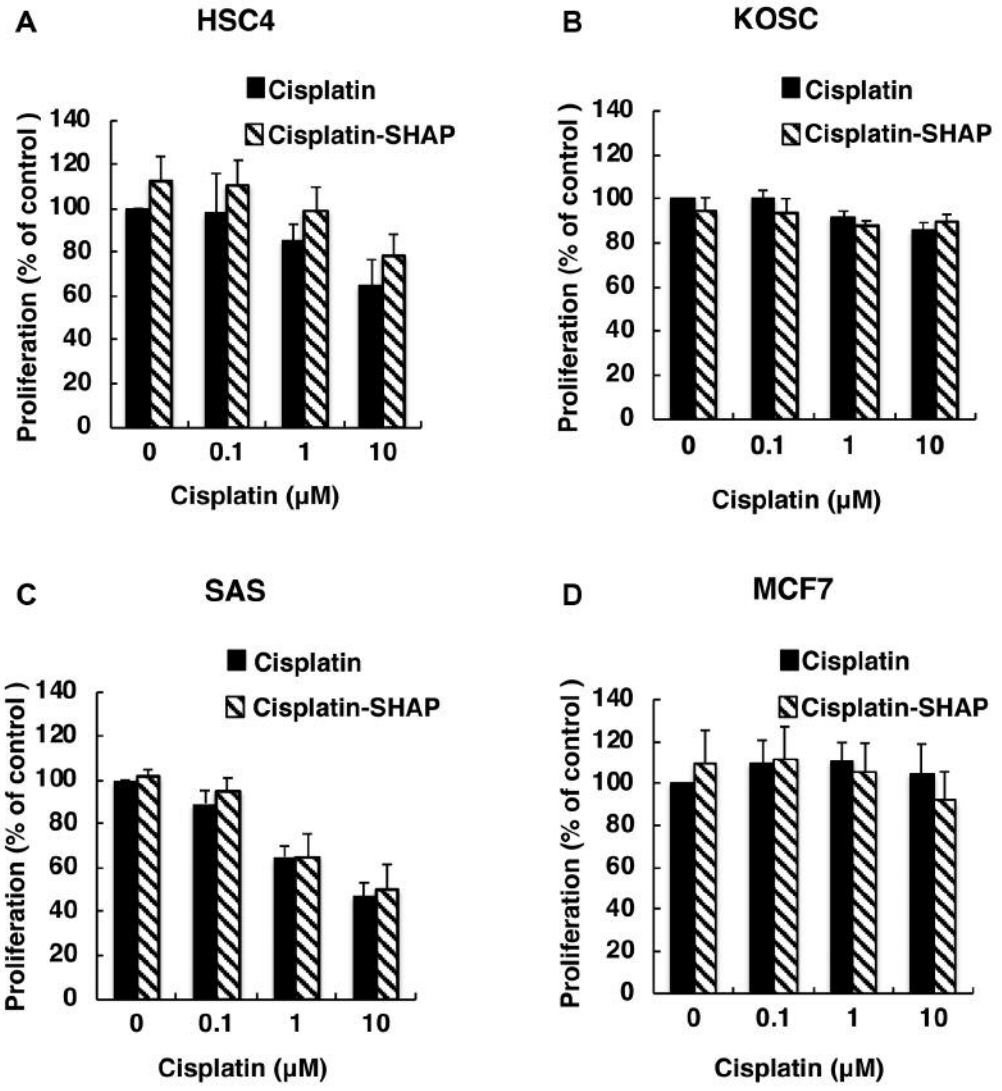

Figure 3. *Effect of cisplatin-bearing highly-dispersive calcined hydroxyapatite nanoparticles (nano-SHAP) on cell proliferation of human oral squamous cell carcinoma cell lines HSC4 (A), KOSC (B), SAS (C) and human breast cancer cell line MCF-7 (D). The cells were exposed to increasing concentrations of cisplatin alone or cisplatin-bearing nano-SHAP (1,000 ng/ml) for 72 h, and the effects on cell proliferation were measured using the MTS assay. All experiments were performed independently three times.*

effects of cisplatin alone and cisplatin-bearing nano-SHAP on cell proliferation of any of the four cell lines (Figure 3).

Effect of carboplatin-bearing nano-SHAP on cell proliferation. Carboplatin is a second-generation platinum-containing antitumor agent commonly used to treat tumors. The effects of carboplatin and carboplatin-bearing nano-SHAP on cell proliferation were examined. However, no differences were found between the effects of carboplatin alone and carboplatin-bearing nano-SHAP on cell proliferation of any of the four cell lines (Figure 4).

Discussion

HAP can be loaded with therapeutic molecules as a DDS, but is known to have different properties depending on morphology, size, and surface properties (2, 3). In the present *in vitro* study, we demonstrated that a newly developed, highly-dispersive calcined nano-SHAP with an average grain diameter of 20 nm might be useful as a drug delivery carrier of ZA, but not of cisplatin or carboplatin, to OSCC cells and breast cancer cells. OSCC can directly invade adjacent jaw bone at relatively early stages and

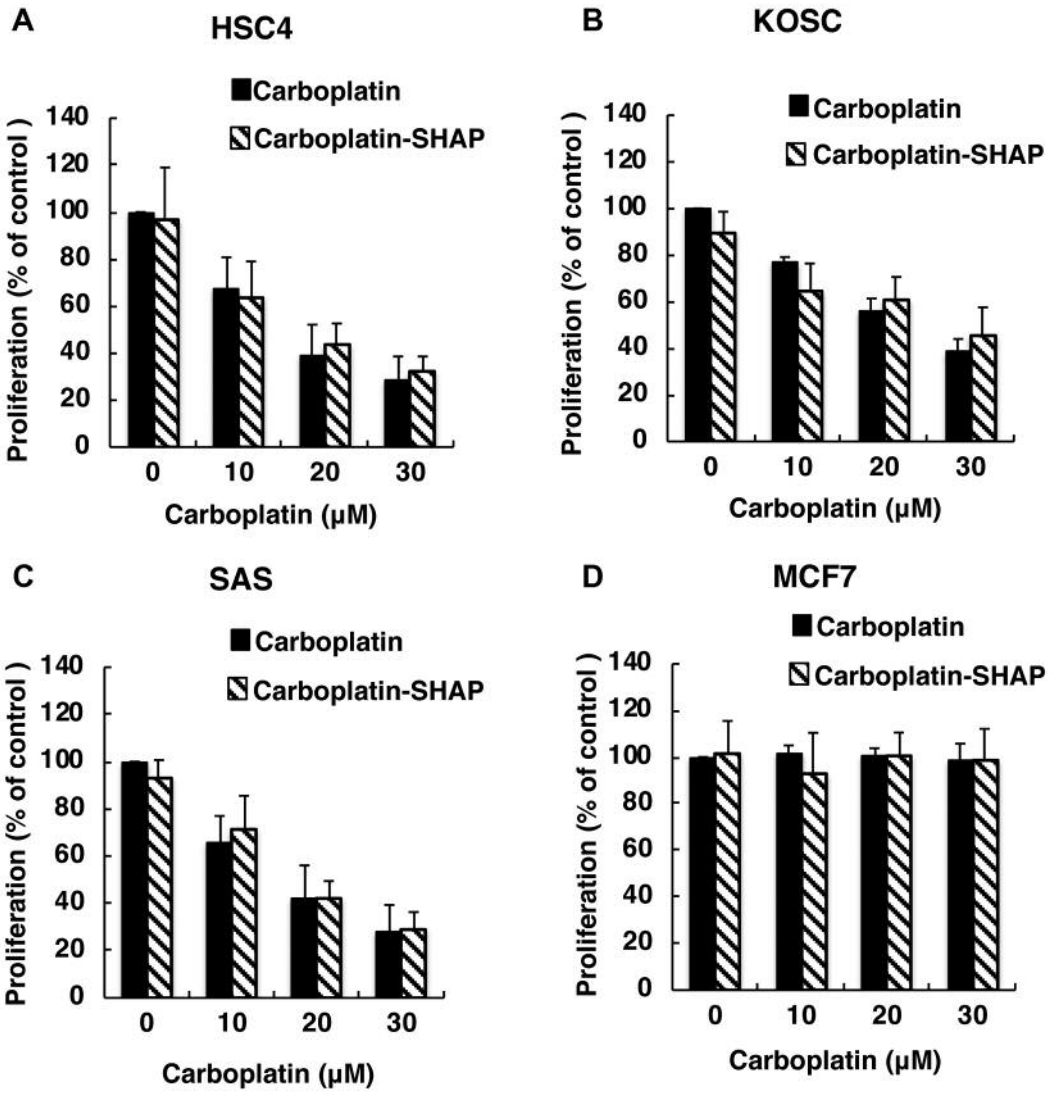

Figure 4. *Effect of carboplatin-bearing highly-dispersive calcined hydroxyapatite nanoparticles (nano-SHAP) on cell proliferation of human oral squamous cell carcinoma cell lines HSC4 (A), KOSC (B), SAS (C) and human breast cancer cell line MCF-7 (D). The cells were exposed to increasing concentrations of carboplatin alone or carboplatin-bearing nano-SHAP (1,000 ng/ml) for 72 h, and the effects on cell proliferation were measured using the MTS assay. All experiments were performed independently three times.*

breast cancer often metastasizes to bone. It has been reported that ZA, a therapeutic agent for osteoporosis and cancer bone metastasis, directly inhibits tumor cell proliferation at peak plasma concentrations following intravenous administration of the standard dose (4 mg). However, because plasma concentrations rapidly decline after less than 24 h (4, 8, 10), a new approach for inhibiting tumor cell proliferation at low concentrations of ZA is needed. Previously, we reported *in vitro* synergistic effects of ZA and calcium on the viability of human OSCC and human skin keratinocytes (4, 8). It is known that BPs, including ZA, have a high affinity for bone mineral because of their backbone P-C-P structure and ability to chelate calcium ions (11). Bone is mainly composed of HAP with a structural formula of $Ca_{10}(PO_4)_6(OH)_2$ (1, 2). Therefore, we investigated the effect of ZA-bearing nano-SHAP on cell proliferation. As expected, ZA-bearing nano-SHAP significantly inhibited cell proliferation relative to ZA alone in OSCC and breast cancer cell lines. HAP is sensitive to pH and can be degraded into calcium and phosphorous elements under weak acidic conditions (12). Therefore, we thought that after ZA-bearing nano-SHAP was taken into cells by endocytosis, it would be dissolved in the intracellular acidic environment and ZA would be released.

Platinum-based drugs have been used to treat various cancer types for decades, including ovarian, cervical, head and neck, and non-small-cell lung cancer. Although platinum-based drugs exhibit strong anticancer activity, they can have serious side-effects. Therefore, a DDS should increase drug concentration to the tumor while reducing the side-effects. Cisplatin is a first-generation platinum agent (13). The specific properties of the drugs and morphology of nano-SHAP were found to affect the adsorption of drug molecules. Positively charged cisplatin was strongly adsorbed to nano-SHAP, whereas cisplatin adsorption was favored at the needle-shaped HAP surface (3). However, cisplatin-bearing nano-SHAP did not inhibit cell proliferation compared with cisplatin alone in any of the cell lines. This may indicate difference in morphology of nano-SHAP or cell type. Furthermore, information on cisplatin concentration in nano-SHAP and the release rate of cisplatin from nano-SHAP is lacking.

Carboplatin is a second-generation platinum agent that exhibits strong antitumor activity (13). To our knowledge, there is only one *in vivo* report on carboplatin-loaded hydroxyapatite, which was the spherical type with a mean diameter of 36.1 μm, and it prolonged the mean survival time in rats with AH130 peritoneal carcinomatosis. It is not clear why our *in vitro* results and their *in vivo* results differed, but it may relate to HAP shape and size, cell type, or loaded condition.

In conclusion, our results suggest that nano-SHAP may be effective as a drug delivery carrier for ZA. Further studies are required to clarify this possibility.

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

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