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Combined therapeutic efficacy of ^{188}Re-liposomes and sorafenib in an experimental colorectal cancer liver metastasis model by intrasplenic injection of C26-*luc* murine colon cancer cells

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Abstract. Rhenium-188 (^{188}Re) displays abundant intermediate energy β emission and possesses a physical half-life of 16.9 h. Sorafenib is an orally available multikinase inhibitor that targets Raf kinases and vascular endothelial growth factor receptors (VEGFRs). Sorafenib has demonstrated preclinical and clinical activity against several types of tumors, such as renal cell and colorectal carcinoma. In this study, we investigated the efficacy of radiotherapeutics of ^{188}Re-liposomes combined with sorafenib in a C26-*luc* metastatic colorectal liver tumour mouse model. Liver metastases were established by intrasplenic injection of C26-*luc* murine colon cancer cells. Based on the results of the toxicity assessment, an administration dose of 80% the maximum tolerated dose was selected. ^{188}Re-liposomes were administered on day 1, when metastases of several hundred micrometers in diameter were observed. In the combination therapy group, 10 mg/kg sorafenib (co-developed and co-marketed by Bayer and Onyx Pharmaceuticals as Nexavar) was administered every other day for 1 week and the survival of mice was assessed. The tumor growth was more significantly inhibited in the ^{188}Re-liposome plus sorafenib group compared with the ^{188}Re-liposome alone, sorafenib alone and untreated normal saline groups (P=0.0000). Furthermore, ^{188}Re-liposomes combined with sorafenib achieved higher survival rates compared with the ^{188}Re-liposome alone, sorafenib alone and untreated normal saline groups (P=0.0000). These results support the use of combined radio-chemotherapy with ^{188}Re-liposomes plus sorafenib as a viable treatment option in the adjuvant setting for liver metastases of colorectal cancer.

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Introduction

Colorectal cancer is the third most common type of cancer worldwide, the second leading cause of cancer-related mortality in humans and the most common type of cancer in the Western world. At the time of diagnosis, ~30% of patients have developed distant metastases, which predominantly occur in the liver. Surgical removal of the tumor remains the only curative approach (1,2). Of all affected patients ~50% develop liver metastases (3) and advanced tumor stage with metastasis is among the main causes of the high mortality rate. Over the last few years, the survival rates for colorectal cancer have further increased due to multimodality treatment concepts, particularly in Union for International Cancer Control stage III and IV patients. In parallel to these modern multimodality treatment concepts, novel and promising concepts, including immunotherapeutic strategies, are actively being investigated to further improve the clinical outcome.

The 5-year survival of patients undergoing hepatic resection was reported to be ~30%, compared with ~10% among patients without hepatic resection (4).

Ionizing radiation (IR) therapy is considered to be an effective local cancer treatment, which eliminates cancer as well as other cells within the tumor stroma. IR induces a variety of DNA lesions, of which DNA double-strand breaks (DSBs) are the most biologically important, since unrepaired or misrepaired DSBs may lead to genomic instability and cell death. IR treatment results in the activation of several DNA damage response molecules, such as ataxia teleangiectasia mutated kinase (ATM), ataxia teleangiectasia and Rad3-related protein (ATR) and catalytic subunit of DNA-dependent protein kinase. ATM and ATR are large, >300-kDa protein kinases that, upon activation, phosphorylate numerous substrates and trigger repair or apoptosis, necrosis, mitotic catastrophe and stress-induced premature senescence (5-9).

Currently, applying nanocarriers for improving cancer diagnostics and therapeutics poses emerging opportunities and challenges (10,11). Liposomal drugs, such as pegylated liposomes, may be designed to improve the pharmacological and therapeutic index for cancer therapeutics. However, the limited distribution of doxorubicin in solid tumors leads to drug resistance, thus weakening the response to chemotherapy (12).

There are considerable developments on improving the therapeutic efficacy, reducing the side effects and overcoming the drug resistance of multiplex nanoliposomes.

Internal radiotherapy with nanoliposomal (range, 100 nm) delivery of radionuclide or chemotherapeutic payloads may be selectively targeted at the tumor, while reducing non-specific accumulation (13). Rhenium-188 (^{188}Re) emits a 155-keV γ-photon and a 2.12-MeV β-particle suitable for nuclear imaging and targeted radionuclide therapy. We previously investigated the biodistribution, pharmacokinetics and single-photon emission computed tomography/computed tomography imaging following intraperitoneal and intravenous administration of ^{188}Re-liposomes in C26 colon carcinoma ascites and solid tumor animal models (14,15).

Sorafenib is an orally available multikinase inhibitor that targets Raf serine/threonine kinases (Raf-1, wild-type B-Raf and B-Raf V600E), vascular endothelial growth factor receptor (VEGFR)-1, -2 and -3, platelet-derived growth factor receptor (PDGFR)-β and Flt3, c-Kit and p38 tyrosine kinases. Sorafenib has a dual action that targets serine/threonine and receptor tyrosine kinases, inhibiting i) the Raf cascade, preventing the downstream mediation of cell growth and proliferation; and ii) the VEGFR-2,-3/PDGFR-β signalling cascade, inhibiting the activation of angiogenesis. Sorafenib acts by inhibiting tumor growth and disrupting tumor microvasculature through antiproliferative, antiangiogenic and proapoptotic effects (16-19). Sorafenib has demonstrated preclinical and clinical activity against several types of tumors, such as renal cell, hepatocellular and colorectal carcinoma (20-29).

Recent progress in the identification of master tumorigenesis signaling pathways and protein kinases has led to the development of novel targeted anticancer drugs. Sorafenib has the potential to synergize with radiation through several mechanisms, including proliferation inhibition of tumor cells, vascular normalization of tumors and interference with intracellular signaling pathways, which may affect the growth and metastatic potential of tumors. Sorafenib administered in combination with radiotherapy may eliminate more tumor cells. There is a strong biological rationale to combining radiation with sorafenib and it was effective in treating mice with metastatic colorectal cancer (29,30). In this study, the tumor inhibitory effect of ^{188}Re-liposomes combined with sorafenib on C26-*luc* metastatic colorectal liver tumours was evaluated.

Materials and methods

Materials. The tungsten-188 (^{188}W)/^{188}Re generator was purchased from Oak Ridge National Laboratory (Oak Ridge, TN, USA). Elution of the ^{188}W/^{188}Re generator with normal saline provided solutions of carrier-free ^{188}Re as sodium perrhenate ($NaReO_4$). The pegylated liposome (Nano-X) was provided by Taiwan Liposome Company (Taipei, Taiwan). N,N-bis (2-mercaptoethyl)-N',N'-diethylethylenediamine (BMEDA) was purchased from ABX (Radeberg, Germany). Stannous chloride ($SnCl_2$) was purchased from Merck KGaA (Darmstadt, Germany). Glucoheptonate (GH) powder was purchased from Sigma-Aldrich (Bangalore, India). PD-10 column was purchased from GE Healthcare (Uppsala, Sweden). All other chemicals were purchased from Merck KGaA. RPMI-1640 cell culture medium and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). Nexavar was obtained from Bayer HealthCare Pharmaceuticals (Montville, NJ, USA).

Cell cultures and animal model. The C26 murine colon carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). This cell line was transfected with the luciferase gene as reporter gene (C26-*luc* cells). The C26-*luc* cell line stably expresses the firefly luciferase gene. C26-*luc* was grown in RPMI-1640 medium supplemented with 10% (v/v) FBS and 2 mM L-glutamine at 37˚C in 5% CO_2. Cells were detached with 0.05% trypsin/0.53 mM EDTA in Hanks' balanced salt solution. Male BALB/c mice were obtained from the National Animal Center of Taiwan (Taipei, Taiwan), with food and water being provided *ad libitum* in the animal house of the Institute of Nuclear Energy Research (INER). The animal research protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the INER.

Liver metastasis model. A liver metastasis model was established in BALB/c mice. The mice were anesthesized and a small incision was made through the skin over the spleen after shaving. The spleen, visible through the abdominal wall, was grasped and a small incision was made over the tip. C26-*luc* cell suspension (30 µl) was injected through a 29-gauge needle into the parenchyma of the spleen. The spleen was removed 2 min later and the incision in the skin was closed. Seven to ten days later, several metastases were identified, often fused with one another.

Preparation of ^{188}Re-liposomes. The labeling method of ^{188}Re-liposomes was as previously described (27-29). Briefly, BMEDA and $SnCl_2$ were used as the reductants and GH was used as an intermediate ligand to form ^{188}Re-SNS/S complexes. BMEDA (5 mg) were pipetted into a glass vial. A volume of 0.5 ml of 0.17 mol/l GH dissolved in a 10% acetate solution was added, followed by the addition of 120 µl (10 µg/µl) of $SnCl_2$. After flushing the solution with N_2 gas, ^{188}R of highly specific activity was added. The vial was sealed and heated in water bath at 80˚C for 1 h. The pegylated liposomes had an average particle size of ~89.46±26.18 nm. Nano-X pegylated liposomes (1 ml) were added to the ^{188}Re-BMEDA (600-740 MBq) solution and incubated at 60˚C for 30 min. ^{188}Re-liposomes were separated from free ^{188}Re-BMEDA using an PD-10 column (GE Healthcare) eluted with normal saline. Each 0.5-ml fraction was collected into a tube. The opacity of pegylated liposomes was employed to visually monitor the collection of ^{188}Re-liposomes. The labeling efficiency was determined using the activity in pegylated liposomes after separation divided by the total activity prior to separation.

Therapeutic efficacy. Treatment was initiated 7-10 days after intrasplenic cell inoculation. A total of 32 BALB/c C26-*luc* tumor-bearing mice were randomly divided into four groups, (n=8 per group) and one group was randomly selected as the control. To confirm the metastasis of tumor cells to the liver, liver tissue was isolated on day 10 post-implantation and *ex vivo* images were captured. Single-dose treatments with ^{188}Re-liposomes were performed on day 1 and triple-dose treatments with Nexavar (10 mg/kg) were performed once every

other day for one week on days 3, 5 and 7. Bioluminescence images were captured on days 1 and 15. Prior to the *in vivo* imaging, the mice were anesthetized with isoflurane. D-luciferin solution was subsequently injected intraperitoneally (150 mg/kg). The mice were imaged using a Xenogen IVIS® 100 small animal imaging system (Caliper Life Sciences, Hopkinton, MA, USA). Excitation (λ_{ex}=710-760 nm) and emission (λ_{em}=810-875 nm) filters were used. Identical illumination settings, including exposure time (10 sec), binning factor (8), f-stop (1) and fields of view (25x25 cm), were used for all image acquisitions. Fluorescent and photographic images were acquired and merged. The images were acquired and analyzed using Living Image® 2.0 software (Caliper Life Sciences). The fluorescence signal intensity of the abdominal region was quantified by creating an circular region of interest (ROI) using Living Image® 2.0 software.

Results

Labeling efficiency of ^{188}Re-liposomes. The encapsulation efficiency of ^{188}Re-BMEDA in pegylated nanoliposomes was 79.2±3.7%. The radiochemical purity of ^{188}Re-liposomes exceeded 95%. The average particle size of ^{188}Re-liposomes was similar to that prior to ^{188}Re-BMEDA encapsulation.

Bioluminescence imaging for monitoring therapeutic response. The therapeutic responses were monitored by bioluminescence imaging prior to and twice a week following drug treatment (Fig. 1A). Significant suppression of tumor growth was observed with the use of ^{188}Re-liposomes. The most significant tumor inhibition was achieved with the combination therapy using sorafenib followed by radiotherapy with ^{188}Re-liposomes. In this study, the normal saline group was used as control for comparison purposes. The photon counts from the bioluminescence imaging were collected and measured from the ROIs of the tumor sites. The mean photon flux of all the treatments correlated with tumor size. The results demonstrated that the mean photon flux of the control group increased rapidly ($2.2x10^8$±$1.4x10^8$ ph/sec) compared with the group treated with ^{188}Re-liposomes ($4.0x10^7$±$2.1x10^7$ ph/sec) at day 15 after treatment. The mean photon fluxes, as a function of time after initiation of the various treatments, are shown in Fig. 1B and the survival curves for the different treatment groups are compared in Fig. 2. At the end of the experiment (41 days after therapeutics administration), 6 mice (75%) treated with ^{188}Re-liposomes plus sorafenib (P=0.000) and 5 mice (62.5%) treated with ^{188}Re-liposomes alone (P=0.000) remained alive. These results confirm that, among all treatments, the greatest tumor control was achieved by the combination of radiotherapy and chemotherapy.

Discussion

Sorafenib is hypothesized to affect tumor growth by directly inhibiting tumor cell proliferation, promoting apoptosis and inhibiting tumor angiogenesis, leading to tumor stasis with occasional tumor regressions. This mechanism of action usually precludes drugs such as sorafenib as single-agent treatment for the majority of solid tumors, since optimal benefits are achieved when combined with conventional chemotherapeutic agents and/or radiotherapy. The combination of sorafenib with radiation was previously described in a variety of human tumor cell lines *in vitro* and *in vivo*. Plastaras *et al* (30) observed that sorafenib exhibits a broad range of antigrowth activity in viability assays in several human tumor cell lines and may also selectively induce apoptosis in some of these cell lines. Sorafenib slows cell cycle progression and prevents irradiated cells from reaching and accumulating at G2-M phase. Radiation treatment followed sequentially by sorafenib was found to be associated with the greatest tumor growth delay (30), whereas concurrent treatment with radiation and sorafenib was not superior to radiation alone. In our study, the group of ^{188}Re-liposome treatment followed sequentially by sorafenib was found to achieve a higher survival rate compared with the ^{188}Re-liposome only, sorafenib only and normal saline control groups.

IR is used as a primary treatment for several types of cancer. Exposure of carcinoma cells to low doses of IR was shown to cause DNA damage and rapid activation of p53, ATM, ATM- and Rad3-related proteins, which further activate growth factor receptors in the plasma membrane (31-34). The ATM/p53 pathway, the mitogen-activated protein kinase (MAPK) cascade and the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pathway are some of the pathways that are activated in response to radiation, affecting long-term cell survival. Cell signaling through the MAPK pathway may result in the expression of cyclin D1 and cell cycle progression through the G1/S checkpoint. Cyclin D1 is a component of the core cell cycle machinery. Abnormally high levels of cyclin D1 are detected in several types of human cancer (35,36). Kim *et al* (23) reported that exposure of colon cancer cells to sorafenib combined with irradiation resulted in increased radiation-induced cytotoxicity. While radiation induced the expression of cyclin B1, sorafenib inhibited cyclin B1 expression. Sorafenib also attenuated cyclin B1 expression when combined with radiation. Sorafenib was shown to inhibit cell cycle progression via the downregulation of cyclin B1, leading to failure of the cells to undergo the transition from the G2 to the M phase. The combination of radiation with sorafenib was shown to reinforce radiation-induced mitotic arrest by attenuating cyclin B1 (23).

In a study conducted by Plastaras *et al* (30), HCT116 tumor-bearing mice were irradiated with four fractions of 3 Gy/day, followed by 7 days of 60 mg/kg/day sorafenib and it was observed that radiation treatment followed sequentially by sorafenib achieved a more significant tumor growth delay compared to radiation alone or concurrent treatment (30). Suen *et al* (22) investigated the combination effect of sorafenib and radiation using two human colorectal cancer cell lines, HT29 and SW48, and observed that radiation treatment followed sequentially by sorafenib treatment exhibited synergistic cytotoxicity in HT29/tk-*luc* cells, with increased tumor cell apoptosis. NF-κB activation induced by radiation may be reduced by sorafenib (22). Kuo *et al* (27) reported that the combination of sorafenib and radiation achived the maximum tumor growth inhibition compared to sorafenib alone or radiation alone. Sorafenib and radiation act synergistically in the treatment of human colorectal

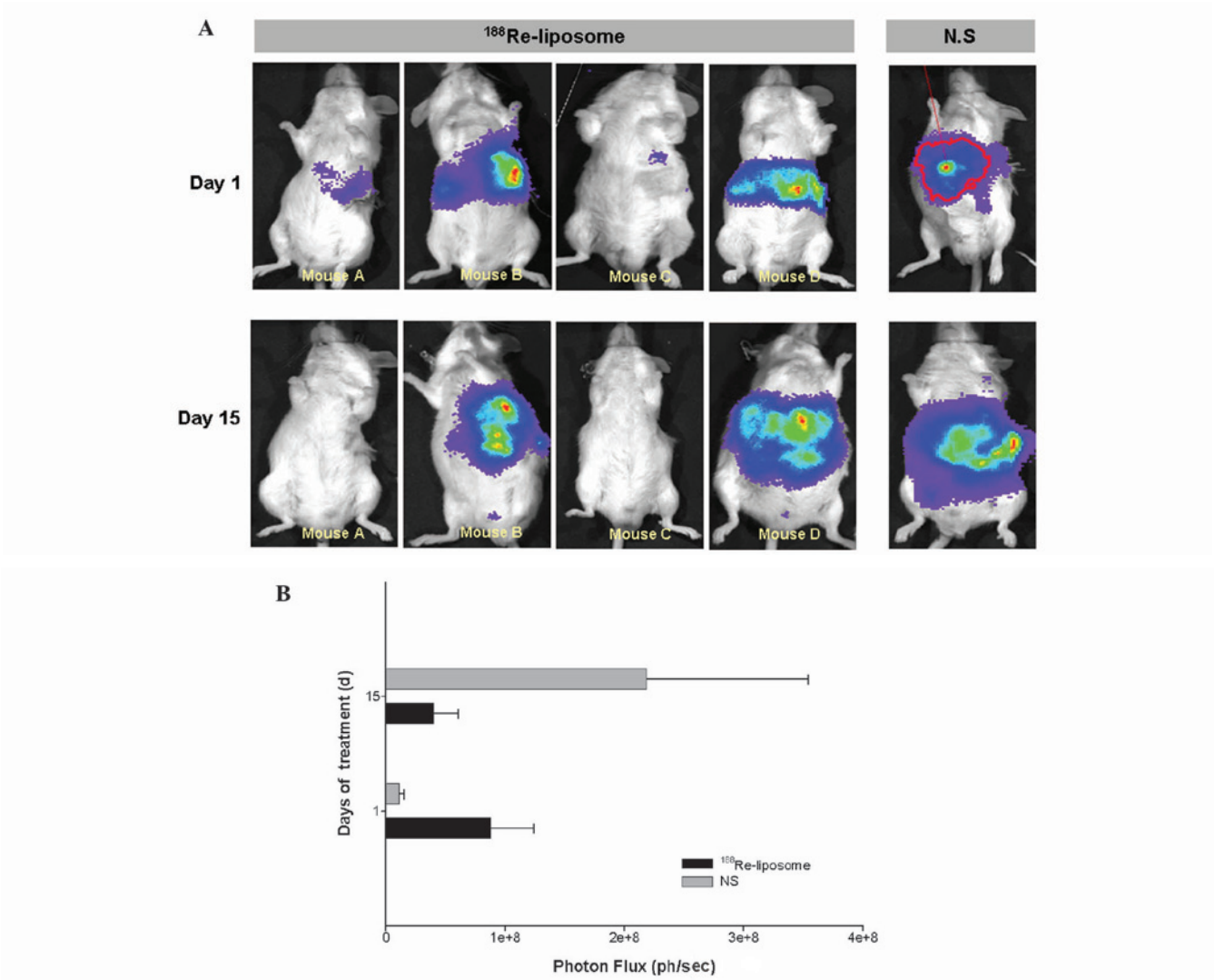

Figure 1. Photon flux distribution of the tumors. (A) The *in vivo* bioluminescence imaging of C26-*luc* tumor-bearing BALB/c mice. C26-*luc* tumor cells were transplanted into the male BALB/c mice, followed by intravenous injection of ^{188}Re-liposomes or normal saline at day 1. Significant therapeutic efficacy was observed in the ^{188}Re-liposome groups. (B) The ^{188}Re-liposome groups exhibited a lower level of photon distribution, which corresponds to tumor growth inhibition. Data are expressed as means ± SEM.

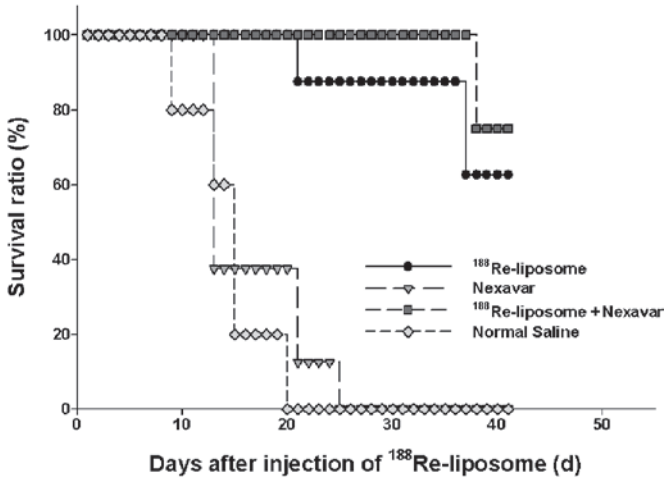

Figure 2. Survival curve for BALB/c mice implanted with C26-*luc* murine colon tumor cells by intrasplenic injection after administering ^{188}Re-liposome (29.6 MBq), Nexavar (10 mg/kg, once every other day for 1 week) or ^{188}Re-liposome (29.6 MBq) combined with Nexavar (10 mg/kg, once every other day for 1 week).

carcinoma. This synergistic action is mediated through the inhibition of radiation-induced NF-κB expression and its regulated downstream gene products (27). In this study, the C26-*luc* tumor-bearing mice were treated once every other day for 1 week with 10 mg/kg sorafenib by gavage 24 h after ^{188}Re-liposome treatment and were continuously treated for 1 week post-irradiation. The results demonstrated that the optimal tumor growth control and survival ratio was achieved with the combination treatment vs. sorafenib alone or radiation alone. Radiation activates the DNA binding of NF-κB and results in the increase of cyclin D1 and cyclin B1, an effect which is suppressed by sorafenib. Therefore, the sequential administration of sorafenib may be an effective cancer treatment schedule when combined with radiation treatment.

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