Phenylboronic Acid Conjugated to Doxorubicin for the Treatment of Hepatocellular Carcinoma Through Transcatheter Arterial Chemoembolization

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Research

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Abstract

Background: Anticancer strategies using nanocarrier systems via the enhanced permeability and retention (EPR) effect and tumor targeting have been explored in various cancers. In previous studies, the anticancer effect of polymerized phenylboronic acid-conjugated doxorubicin (pPBA-Dox) nanocomplexes was confirmed in various cancers, and their anticancer effect and tumor targeting ability was confirmed in hepatocellular carcinoma (HCC).

This study aimed to determine the anticancer effect and changes in the liver immune cell population and function after pPBA-Dox nanocomplex infusion through transcatheter arterial chemoembolization (TACE), which is a locoregional therapy (LRT), in HCC. TACE was performed in a rat liver cancer model, and the anticancer effects, immune cell populations and functional changes were confirmed after 1 week. Magnetic resonance imaging (MRI) and flow cytometry (FACS) were performed to analyze the anticancer effect and immune cell population and function.

Results: In HCC, the infusion of pPBA-Dox nanocomplexes through TACE had a stronger anticancer effect than conventional doxorubicin (Dox) and it promoted the infiltration and activation of CD4⁺ and CD8⁺ T cells in the liver.

Conclusions: This study provides insight into novel targeted therapies using nanocomplexes for the treatment of HCC.

Background

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the fourth-highest most common cancer-related death with a very poor survival rate. The prevalence of HCC has been higher in sub-saharan Africa and Southeast Asia, but its incidence rate is steadily increasing in Western countries (1, 2).

Currently, The Barcelona Clinic Liver Cancer (BCLC) staging system which is based on tumor stage, liver functional status, physical status, and cancer-related symptoms, has been widely used when deciding on the treatment of HCC. There are various treatment modalities for HCC depending on the BCLC system, such as hepatic resection, transplantation, transarterial chemoembolization (TACE), local ablation therapy, and systemic therapy. In the asymptomatic HCC patients with unresectable lesions without extrahepatic metastasis, TACE is considered to be the first-line treatment (3). However, TACE has a 52.5% objective response rate (ORR) (4) and a median survival of 26–40 months (5, 6), and there are significant differences in tumor burden and liver function depending on the patient, making it difficult to achieve the same effect through TACE. Therefore, to improve the clinical outcomes, TACE drugs with fewer side effects and strong anticancer effects are necessary.

Drug delivery systems have emerged as alternative technology platforms for cancer treatment. To date, various kinds of nanocarriers, such as microspheres, nanoparticles, micelles, dendrimers, liposomes, and

polymers, have been explored for the treatment of cancers and noncancerous diseases. They can take advantage of the enhanced permeability and retention (EPR) effect to cross the vascular barrier and accumulate in the tumor microenvironment, which enhances the therapeutic effects of drugs and reduces their side effects. However, the materials used in drug delivery systems such as polymers are often recognized as foreign materials when entering the body, and the body attempts to attack foreign materials, which means that they invoke an immune or inflammatory response. As a result, nanoparticles will be targeted by the immune system and reduce the drug delivery efficiency (7–9).

Phenylboronic acid (PBA) is known to form reversible complexes with *cis-diol*-containing compounds, such as various monosaccharides. PBA can accurately recognize the sialic acids (SAs), which are commonly found as the terminal monosaccharides of the glycans that cover the cell surface of mammalian cells. Regulation of sialylation adding SAs during glycoprotein biosynthesis is regulated by expression of sialyltransferase. The expression of sialyltransferase is reported to be high in the liver, spleen and kidney, and less than 5% in other organs (10). Moreover, abnormal sialylation is associated in malignant properties, including invasion and enhanced cell survival. Therefore, a recent study used these properties to PBA-installed micellar nanocarriers incorporate anticancer drugs to target sialylated epitopes overexpressed on tumor cells (11–14).

Recently, we reported on the characterization of self-assembled nanocomplexes generated *via* utilizing the hydrophobic interaction between polymerized PBA (pPBA) and the anticancer drug doxorubicin (Dox), which intercalates with DNA and inhibits topoisomerase α leading to inhibit cancer cell proliferation (15). Dox is a drug most commonly used in TACE and has a hydrophobicity and cationic charge that result from the 1,3-diol confirmation in its structure, which is a specific chemical property and thus binds strongly with PBA through the boronic ester. These pPBA-Dox nanocomplexes were expected to have a more potent anticancer effect by passive targeting through EPR effect and active targeting from residual PBA moieties. And these nanocomplexes are easily released into the cell membrane by binding affinity of PBA depends on the pH.

Therefore, we have previously confirmed that pPBA-Dox nanocomplexes have a more potent efficient anticancer targeting properties and sustained long-term release patterns in breast cancer and liver cancer through *in vitro* and *in vivo* experiments (15, 16). However, systemic administration of drugs may cause side effects in other organs. Therefore, with the aim of minimizing drug side effects in other organs, we evaluated the anticancer effect of pPBA-Dox nanocomplexes through TACE, which is a direct drug infusion method used to treat liver cancer.

In this study, we evaluated the effectiveness of pPBA-Dox nanocomplexes in a rat liver cancer model using TACE. We evaluated the minimal off-target side effects of pPBA-Dox nanocomplexes and confirmed their improved anticancer effects after TACE. In addition, flow cytometry analysis confirmed changes in the liver immune response induced by the pPBA-Dox nanocomplexes.

Results

Confirmation of the rat liver cancer model through clinicopathological analysis

In 8-week-old Sprague-Dawley (SD) rats, McA-RH7777 cells were injected into the left lobe liver. To prevent spontaneous regression, 20 mg/kg cyclosporin A was injected subcutaneously a day before the cell injection and then daily until 4 days later. Fourteen days after the cell injection, the tumor size was confirmed by MRI, and TACE was performed. One week later, the tumor size confirmed the efficacy of the drug (Fig. 1A).

To confirm the formation of a rat liver cancer model, the hematologic levels were checked in the rat serum. The ALT of the liver cancer model was 261 ± 24.98 U/L, which was higher than that of the normal group (58.67 ± 2.84). The levels of creatinine between the liver cancer model and the normal group were all within the normal range (0.1 to 0.6 mg/dL), and there was no significant change (Fig. 1C). In addition, we further evaluated the liver cancer model serologically by measuring the AFP levels by ELISA (Fig. 1D). In the liver cancer model, the AFP was 452.6 ± 24.73 ng/ml, which was significantly higher than that of the normal group (57.31 ± 7.38 ng/ml). When the size of the tumor was confirmed through MR imaging, at 14 days after the cell injection, the length of the tumor was 14.29 ± 1.6 mm and its volume was 662.3 ± 49.81 mm³ (Fig. 1E, F). HCC in the liver was identified by H&E staining (Fig. 1G).

Interestingly, 8 weeks after the cell injection, the AFP increased to 714.7 ± 41.07 mg/dL, and the tumor length increased to 67.35 ± 1.45 mm. Lung metastasis was observed in 2 out of 3 rats, but there was no metastasis to the other organs (heart, kidney and spleen) (Supplement Fig. 1).

Drug infusion into liver cancer via TACE

TACE was performed in a total of 27 SD rats, and all rats had a tumor ≥ 10 mm on baseline MRI (Fig. 2A). The rats were divided into the pPBA-Dox nanocomplexes (n = 7), Dox (n = 6), pPBA (n = 6), and no-treatment (n = 8) groups. Each drug was infused at a concentration of 2 mg/kg pPBA-Dox nanocomplexes (containing 1 mg/kg Dox equivalent), 1 mg/kg Dox, or 2 mg/kg pPBA. After chemoembolization, none of the rats exhibited any complications, including neurological deficits, pneumonia, or bleeding.

Anticancer effect of pPBA-Dox nanocomplexes via transarterial chemoembolization

As in previous studies (16), the size of the liver cancer in the group treated with pPBA-Dox nanocomplexes decreased compared with that in the group treated with Dox. As shown in Fig. 3A, in the MRI image, the tumor size after TACE was smaller in the pPBA-Dox and Dox groups than before TACE, while in the no-treat and pPBA groups, the tumor size increased. For the tumor size in each group, the pPBA-Dox nanocomplexes group was 289.6 ± 50.9 before TACE and then it decreased to 144.4 ± 59.5 after TACE, while the Dox group decreased from 513.2 ± 83.82 to 444.7 ± 92.33 . The tumor sizes in the

untreated and pPBA groups increased from 638.6 ± 160 to 1886 ± 380.2 and from 831.4 ± 276.8 to 1342 ± 463.6 , respectively (Fig. 3B). The tumor volume reduction ratio ((After-Before)/Before, VRR) (17) in the MRI images was calculated to quantitatively analyze the antitumor activity of each agent (Fig. 3C). The VRR value of the pPBA-Dox nanocomplexes was -0.60 ± 0.1 , which was lower than that of the Dox group (-0.16 ± 0.07), and the tumor was significantly reduced compared with that of the Dox group. The VRR values of the no-treatment and pPBA groups were 3.1 ± 1.2 and 0.8 ± 0.4 , respectively, and the tumors were larger than before.

When comparing the AFP reduction ratio ((After-Before)/Before), the AFP reduction ratio of the pPBA-Dox nanocomplex group was -0.02 ± 0.03 , which was the smallest change in the AFP values before and after TACE, relative to the Dox 0.12 ± 0.08 , pPBA 0.53 ± 0.13 , and no-treatment 0.34 ± 0.09 groups (Fig. 3D). Although the AFP reduction ratio of the pPBA-Dox nanocomplexes and Dox groups did not decrease before and after TACE, the VRR value decreased, demonstrating that the tumor size decreased.

In the case of ALT, the pPBA-Dox nanocomplex group was 61.25 ± 4.7 U/L, which was lower than the no-treatment group ALT value of 261.6 ± 81.06 U/L, and the ALT values of the Dox group (92.83 ± 26.91 U/L) and pPBA groups (84.5 ± 14.1 U/L) were reduced (Fig. 3E). In addition, the levels of creatinine did not differ significantly among the groups, and since all groups were within the normal range of 0.1 to 0.6 mg/dL, any nephrotoxicity caused by the agents could not be confirmed (Fig. 3F).

Although the serum AFP level measurement for 1 week did not necessarily correspond to a decrease in tumor size, AFP did not increase in the group treated with the pPBA-Dox nanocomplexes. However, in the other groups, most of the rats had elevated AFP after TACE. These results suggest that pPBA-Dox nanocomplexes have remarkable anticancer potential against HCC through TACE.

Based on the H&E staining of the liver after TACE, the tumors in the pPBA-Dox nanocomplex and Dox groups showed necrosis, and it was confirmed that the immune cells had infiltrated around the tumor (Fig. 3G). To confirm the role and function of the infiltrated immune cells in the liver, the anticancer effect of the immune cells was confirmed through FACS analysis.

Increased T cell population after TACE

To further verify the effects of TACE treatment on the immune population and function, the immune status of the rats was examined after TACE treatment. CD4⁺, CD8⁺, CD69, Ki-67 and PD-1 were selected as indices to evaluate the immune function. T lymphocyte subpopulations were assessed in the liver, spleen, and peripheral blood mononuclear cells (PBMCs) using FACS strategies (Fig. 4A).

Liver tissue was collected from the peritumor (Peri-T) region of the same lobe, and the numbers of CD4⁺ and CD8⁺ T cells per 1 g liver tissue were compared and analyzed for each group. After pPBA-Dox nanocomplex treatment through TACE, the CD4⁺ T cells per 1 g were 61820 ± 8980 cells, which was increased compared with those of the untreated (21000 ± 4010 cells), pPBA (29760 ± 8070 cells), and Dox (37050 ± 8350 cells) groups. In addition, the number of CD8⁺ T cells in the Peri-T region of the liver was

44810 ± 5160 cells in the pPBA-Dox nanocomplex group, which was increased compared with that of the untreated (10830 ± 2150 cells), pPBA (11690 ± 3700 cells), and Dox (2839 ± 3903 cells) groups (Fig. 4B).

In the spleen, there was a change in the number of CD4⁺ T cells according to the drug. In the Dox group, the number of CD4⁺ T cells increased to 1729231 ± 308525 cells compared with the untreated group (1099125 ± 308823 cells), but in the pPBA-Dox nanocomplex group, the number decreased to 919286 ± 163954 cells. The CD8⁺ T cells in the spleen demonstrated no significant differences among the groups, but the number of CD8⁺ T cells increased in the pPBA-Dox nanocomplexes group (505176 ± 115735 cells) and Dox group (642625 ± 139554 cells) compared to the No-treat group (410453 ± 98388 cells) (Supplement Fig. 2A).

In PBMCs, the number of CD4⁺ T cells increased in the pPBA-Dox nanocomplexes (12.79 ± 3.05%) and Dox groups (8.05 ± 1.05%) compared to the untreated group (5.49 ± 0.9%), and it was confirmed that the CD8⁺ T cells increased significantly in the pPBA-Dox nanocomplex group (5.3 ± 1.05%) compared to the Dox (2.45 ± 0.4%) and untreated groups (2.3 ± 0.5%) (Supplement Fig. 2B).

To confirm the increased T cell activity after drug infusion through TACE, we additionally identified CD69, Ki-67 and PD-1, activation markers of T cells (18–20). In the case of CD69, the number of CD4⁺ T cells was 7540 ± 1180 cells, which was significantly increased in the pPBA-Dox nanocomplex group compared with the untreated group (4110 ± 1012 cells). It was also significantly increased when compared to the Dox group with 2578 ± 1112 cells. For the CD8⁺ T cells, the number of CD69-expressing cells was increased in the pPBA-Dox nanocomplex group (6390 ± 1621 cells) compared to the untreated (2797 ± 731 cells) and Dox (2256 ± 574 cells) groups (Fig. 4C).

In the case of Ki-67, the number of Ki-67 expressing cells was increased in the pPBA-Dox nanocomplexes group (14220 ± 212 cells) compared to the No-treat (8987 ± 2061 cells) and Dox (9023 ± 1090 cells) groups in the CD4⁺ T cells, and the pPBA-Dox nanocomplexes group (14721 ± 2032 cells) had increased Ki-67 expression in the CD8⁺ T cells compared with the No-treat (10334 ± 1432 cells) and Dox (5232 ± 775 cells) groups (Fig. 4D).

In the case of PD-1, there was no difference in the expression of PD-1 between groups for the CD4⁺ T cells. However, in the CD8⁺ T cells, the pPBA-Dox group had a higher number of PD-1-expressing cells (7764 ± 233 cells) than the no-treat (6732 ± 1998 cells) group. Additionally, in the Dox group, 4824 ± 1143 cells expressed PD-1, which increased the number of PD-1-expressing cells compared to that in the untreated group but it was lower than that in the pPBA-Dox nanocomplex group (Fig. 4E).

To confirm the cytotoxic effect of PBMCs and liver T cells, they were cocultured with McA-RH7777 cells at a 5:1 ratio. Both PBMCs and liver T cells stimulated with PHA demonstrated high cytotoxic effects of 19.6 ± 1.24% and 16.68 ± 0.97% in the pPBA-Dox nanocomplex group. It had a higher cytotoxic effect than the no-treatment group (12.56 ± 0.87% and 10.06 ± 0.59%) and the Dox group (13.55 ± 1.13% and 13.72 ± 0.88%) (Fig. 4F).

Discussion

In the present study, we demonstrated that there is an effective anticancer effect and immune response in liver cancer through TACE using pPBA-Dox nanocomplexes.

According to the BCLC guidelines, many HCC patients attempt LRT as the primary treatment method, and 40% of HCC patients attempt TACE as their primary treatment (21). However, there is a significant difference in tumor burden and liver function depending on the patient, making it difficult to achieve the same effect through TACE. Accordingly, systemic chemotherapy has been proposed, and a new paradigm for anticancer treatment has been proposed with the recent development of immune checkpoint inhibitors (ICIs). However, due to the low response rate of ICIs, clinical studies of anticancer treatment through combination treatment with existing treatment methods are actively progressing (22–25).

We verified the anticancer effect of liver cancer through TACE of pPBA-Dox nanocomplexes and confirmed the change in immune cells in the liver according to drug infusion through TACE. To confirm this hypothesis, we established an animal model of TACE. Generally, the rabbit liver cancer model using VX2 tumor cells has been widely used because the size of the rabbit hepatic artery is appropriate for the introduction of microcatheters. The rabbit model has the advantage of being easy to perform angiography with large blood vessels, but it is difficult to handle, and as neoplasm VX2 tumor cells are not hepatocyte-derived tumors, there is a limitation in that they are physiologically different from HCC (26, 27), so this model differs from liver cancer in humans (28). For these reasons, there is a need for rodent HCC models derived from hepatocellular carcinoma appropriate not only for TACE but also for various studies of anticancer therapies.

Rat liver cancer models using two rodent HCC cell lines are widely used. One is a Morris hepatoma model using McA-RH7777 hepatoma cells in Buffalo rats, and the other is a Novikoff hepatoma model using N1-S1 hepatoma cells in SD rats. Although McA-RH7777 cells have a higher tumor induction rate than N1-S1 cells, buffalo rats are not widely available in some countries and are generally more expensive than SD rats. Therefore, an HCC model was established in the widely used SD rats using McA-RH7777 cells with high tumorigenicity, and TACE was attempted using this model (29). The SD rats showed a tumor formation rate of greater than approximately 95%, and the tumor size increased by 14.29 ± 2.78 mm and 662.3 ± 86.28 mm³ 2 weeks after cell injection. In addition, ALT and AFP were also significantly increased. Interestingly, approximately 8 weeks after cell injection, metastasis to the lung was also observed, showing the possibility of this model as a late metastasis model.

After TACE, tumor size and serum analysis according to each drug showed that the pPBA-Dox nanocomplex group had a higher anticancer effect than the Dox group. The tumor size of the pPBA-Dox nanocomplex group decreased more than that of the Dox group. For AFP, a biomarker of HCC, the AFP reduction ratio in the pPBA-Dox nanocomplex groups was -0.02 ± 0.03 , which was significantly reduced compared to the other groups in which AFP was increased. Both ALT and creatinine were within the normal range, suggesting significant results through hematological analysis. Through these results, it

was confirmed that pPBA-Dox nanocomplex infusion through TACE showed a high anticancer effect on liver cancer but did not show hepatotoxicity or nephrotoxicity.

After TACE in the pPBA-Dox nanocomplexes and Dox groups, it was confirmed through H&E staining that tumor necrosis occurred and immune cells infiltrated around the tumor. In addition, there is a report that PBA used as a drug delivery agent also affects the proliferation of PBMCs (30). Therefore, we confirmed the change in the T cell population in the liver after TACE to confirm the possibility of ICIs after TACE. Because of the heterogeneity of liver cancer, it is difficult to achieve a high therapeutic effect with monotherapy, and to overcome this, combination therapy using several treatment methods is being attempted (31, 32). Recently, in many clinical trials, studies of combination therapy using LRT and ICIs have been conducted (33). It is difficult to obtain patient liver tissue after TACE, and some results have confirmed T cell function after radiofrequency in a murine xenograft model, but there is no result confirming any changes in the T cell population and function in the liver after LRT (34). Other than that, there are only studies indirectly confirming changes in the T cell population and function after LRT in PBMCs (23, 35–37). Therefore, this study was able to directly confirm the possibility of ICIs after LRT, especially TACE, by confirming a change in the T cell population in the liver after LRT.

CD4⁺ and CD8⁺ T cells were significantly increased in the pPBA-Dox nanocomplex group compared with the Dox group. As a hypothesis to explain the increase in the immune cells in the pPBA-Dox nanocomplex groups, we suggest that pPBA-Dox nanocomplexes accumulate and remain in tumor cells for a long time, as shown in previous studies (15, 16), which promotes immune modulation and additionally induces necrosis of tumor cells. These effects induce immunogenic cell death (ICD), and dying tumor cells upregulate and release death-associated molecular patterns (DAMPs), such as calreticulin (CRT), adenosine triphosphate (ATP), and high-mobility-group Box 1 (HMGB1) (38). Thereafter, phagocytes are recruited, and the activation of dendritic cells (DCs) loads major histocompatibility complex (MHC) molecules, which eventually act as antigens on T cells and enhance T cell infiltration (39). This promotes the cytotoxic T cell immune response, which promotes tumor death.

In the rat model, the pPBA-Dox nanocomplex group had more CD4⁺ and CD8⁺ T cells than the Dox group after TACE, which means that T cell infiltration was higher in the pPBA-Dox nanocomplex group than in the Dox group. These results suggest that pPBA-Dox nanocomplexes induce more cell death than Dox through tumor-specific targets, thereby promoting T cell infiltration. On the other hand, T cell infiltration did not increase in the pPBA group. These results indicate that the drug delivery nanomaterial pPBA, a foreign material, does not induce an immune response in the liver.

After the pPBA-Dox nanocomplexes were infused via TACE, the CD4⁺ and CD8⁺ T cell populations increased, and the T cells were activated. After TACE, the expression of CD69, Ki-67 and PD-1 in the CD8⁺ T cells was increased in the group infused with pPBA-Dox nanocomplexes in the liver. In addition, the expression of CD69 and Ki-67 in CD4⁺ T cells was high in the pPBA-Dox nanocomplexes. This suggests that activated CD8⁺ T cells secrete cytokines and cytolytic molecules to kill tumor cells, and activated CD4⁺ T cells assist cytotoxic T cells and other immune cells in performing effector functions, thereby

exerting anticancer effects (40–44). These results demonstrated that when pPBA-Dox nanocomplexes were infused through TACE, they affected immune cells and increased the anticancer effect. Similar to the results of the cytotoxicity assay, immune cells isolated from the pPBA-Dox nanocomplex group exhibited higher anticancer effects on tumor cells. During the cytotoxicity assay process, PHA is a nonspecific stimulant of normal lymphocytes and has the disadvantage of stimulating not only cytotoxic T cells but also all immune cells, such as NK cells. This needs to be confirmed in a future study, but there may have been a change in the frequency of immune cells depending on each drug after TACE. Therefore, even if the same stimulation was given to each group with PHA, it is expected that pPBA-Dox nanocomplexes would have a strong cytotoxicity effect.

In the present study, the pPBA-Dox nanocomplexes had a strong anticancer effect when treating liver cancer using TACE and they induced changes in the immune cell population in the liver. This change in immune cells promoted the infiltration and activation of CD4⁺ and CD8⁺ T cells in the liver. In conclusion, our study suggests a targeted cancer therapy strategy using pPBA-Dox nanocomplexes is effective for the treatment of HCC, and it may be a promising candidate anticancer drug.

Materials And Methods

Preparation and properties of the pPBA-Dox nanocomplexes

The pPBA-Dox nanocomplexes were easily constructed according to our previously described method (15, 16). First, pPBA was conjugated *via* boronic ester formation between PBA-NH₂ and pMVEMA, and pPBA was mixed with Dox at a 2:1 molar ratio to prepare the pPBA-Dox nanocomplexes. To further validate the pPBA-Dox nanocomplexes, physicochemical characterization of their pH-responsive drug release and the structural formation of pPBA-Dox nanocomposites was performed following a previously described method (15).

Rat hepatoma cell line

The McA-RH7777 rat hepatoma cell line from the American Type Culture Collection (ATCC, Rockville, MD, USA) was cultured at 37°C under a humidified atmosphere with 5% CO₂. The cells were grown in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% fetal bovine serum (Gibco, NY, USA).

Rat liver cancer model

Male 8-week-old Sprague–Dawley (SD) rats were purchased from Orient Bio Inc., (Seoul, Korea) and housed at 22°C ± 5°C under a 12 h light/dark cycle. SD rats were anesthetized with 1% isoflurane at a 1:2 ratio of O₂:N₂O during tumor implantation. After anesthesia, a midline minilaparotomy was performed, and the left lateral hepatic lobe was exposed. McA-RH7777 rat hepatoma cells were gently injected under the hepatic capsule into the left lateral lobe. A bipolar coagulation electrosurgical unit (I.T. C Co., Daejeon, Korea) was applied to prevent bleeding and reflux of the cells, and then the abdominal incisions were

closed with a two-layer technique. To prevent spontaneous tumor regression, cyclosporin A (20 mg/kg/d; Chong Kun Dang Pharmaceutical, Seoul, Korea) was subcutaneously administered from 1 day before tumor implantation until 4 days after surgery. After 14 days, the tumor growth was monitored using MRI (Agilent Technologies, Santa Clara, CA, USA). All surgical interventions and presurgical and postsurgical animal care were provided in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Survival Surgery provided by the Institutional Animal Care and Use Committee (IACUC) of the School of Medicine, The Catholic University of Korea (approval number: CUMC-2020-0147-03).

Antitumor response by MRI evaluation

MRI examinations were performed before and after treatment to assess the tumor volume after anticancer treatment of the rats. All rats were maintained under anesthesia with 1% isoflurane in a 1:2 mixture of O₂:N₂O during the examinations. MR images were obtained using a 9.4 T/160 mm animal MRI system (9.4T MRI System; Agilent Technologies). Radiofrequency excitation and signal detection were accomplished with a 40 mm millipede volume coil. The imaging protocol included coronal T2-weighted imaging (T2WI) [(TR = 4000 ms, TE = 32.26 ms, slice number = 12, slice thickness = 1 mm, field of view = 50 × 40 mm², matrix = 192 × 128 (no gap))] and axial T2WI [(TR = 4000 ms; TE = 32.26 ms; slice number = 24; slice thickness = 2 mm; field of view = 25 × 40 mm²; and matrix = 128 × 192 (no gap)]. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to determine the tumor size and location from the obtained MR images.

Transarterial chemoembolization (TACE)

TACE was performed by an experienced interventional radiologist. Anesthesia was achieved by injecting a solution of zolazepam (30 mg/kg, Zoletil; Virbac, Carros Cedex, France) and xylazine (10 mg/kg, Rompun; Bayer-Schering Pharma, Berlin, Germany) intraperitoneally. After anesthesia was achieved in the same manner for tumor implantation, the rats were placed on an angiography table, and the neck was shaved. A 2 cm longitudinal paramedian incision was made in the neck. The left common carotid artery was exposed by dissecting the neck muscles and separating the carotid sheath. The distal common carotid artery was ligated using 4 – 0 blue nylon (AILEE Inc., Busan, Korea), and a double-loop sling was formed around the proximal common carotid artery with 4 – 0 blue nylon. The common carotid artery was cannulated by a 24-gauge intravenous catheter (BD Biosciences, San Jose, CA, USA). A 0.014 inch (0.36 mm) guidewire (ASAHI INTECC, Aichi, Japan) was inserted through the cannula, and during the procedure, the aforementioned sling was sufficiently tightened to prevent bleeding at the carotid artery cannulation site. After inserting the guidewire into the left hepatic artery, a 1.5 Fr microcatheter (TAEWOONG medical, Gimpo, Korea) was advanced into the common hepatic artery (Fig. 2A). A common hepatic arteriogram was obtained to identify the proper hepatic artery anatomy by manually injecting contrast media (Visipaque; GE Health care, Chicago, IL, USA) through the microcatheter (Fig. 2B). Angiography was performed with the Mobile C-arm X-ray System (Siemens Healthineers, Erlangen, Germany). To evaluate the anticancer response to the nanocomplexes, the rats were infused with each

drug through a microcatheter into the proper hepatic artery. The microcatheter was then removed, and the common carotid artery was ligated. The neck incision was sutured with a one-layer technique.

Follow-up MRI was conducted 7 days after the TACE procedure. After the last follow-up MRI, all rats were euthanized, and the tumors were sampled immediately.

Serological assays: serum α -fetoprotein (AFP), alanine aminotransferase (ALT) and creatinine levels

To serologically determine the antitumor response of the pPBA-Dox nanocomplexes and the other compounds, the HCC tumor biomarker AFP was measured using a rat Quantikine ELISA Kit (MyBioSource, CA, USA) following the manufacturer's instructions.

In addition, to evaluate the liver and renal toxicities, TACE, serum ALT and creatinine levels were measured using an ALT and creatinine assay kit following the manufacturer's protocol (Vettest 8008 Chemistry Analyzer; IDEXX Lab., UK).

Flow cytometry

Multicolor flow cytometry was performed using the following commercially available antibodies: allophycocyanin (APC)-conjugated anti-rat CD3, phycoerythrin (PE)-cyanine 7 (Cy7)-conjugated anti-rat CD8a (BioLegend, San Diego, CA, USA), V450-conjugated anti-rat CD4 (BD Biosciences), PE-conjugated anti-rat programmed cell death protein 1 (PD-1) (antibodies-online, Aachen, Germany), PE-conjugated anti-rat CD69 (antibodies-online), and PE-conjugated anti-rat Ki-67 (eBioscience, CA, USA). Dead cells were excluded using aquafluorescent LIVE/DEAD dye (Invitrogen, MA, USA). Multicolor flow cytometry was performed using an LSR Fortessa Canto $\square$ instrument (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

Cytotoxicity assay

Peripheral blood mononuclear cells (PBMCs) from the rat liver cancer model were isolated using Ficoll-Hypaque density gradient centrifugation as previously described (45). Immune cells in the liver were isolated after mechanical disruption (46). Isolated rat immune cells and PBMCs were activated by phytohemagglutinin (PHA, 10 μ g/ml). McA-RH7777 cells were cultured in a 12-well plate for 24 hours. Then, activated rat immune cells and PBMCs were cocultured with McA-RH7777 cells at different effector-to-target ratios. After an additional 24 hours of incubation at 37°C, the cytotoxicity was analyzed by staining with TO-PRO-3 iodide (Invitrogen).

Histological analysis

The organ tissues were fixed in 3.7% buffered formalin and then embedded in paraffin wax. Following embedding, serial sections (3–5 μ m thick) were prepared from the paraffin-embedded tissue. The sections were stained with hematoxylin & eosin (H&E).

Statistical analysis

We compared the resulting tumor volume, AFP level and immune population of each group using Tukey's multiple-comparison posttest. Differences between groups were considered significant at a *P value* < 0.05. All data are presented as the mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism 7 software.

Abbreviations

EPR: Enhanced permeability and retention; pPBA-Dox: Polymerized phenylboronic acid-conjugated doxorubicin; HCC: Hepatocellular carcinoma; TACE: Transcatheter arterial chemoembolization; LRT: Locoregional therapy; MRI: Magnetic resonance imaging; FACE: Flow cytometry; Dox: Doxorubicin; BCLC: Barcelona Clinic Liver Cancer; ORR: Objective response rate; PBA: Phenylboronic acid; SAs: Sialic acids; PEI: polyethyleneimine; pPBA: polymerized PBA; SD: Sprague-Dawley rats; AFP: α-fetoprotein; ALT: alanine aminotransferase; APC: allophycocyanin; PE: phycoerythrin; Cy7: cyanine 7; PD-1: programmed cell death protein 1; PBMC: Peripheral blood mononuclear cell; PHA: phytohemagglutinin; VRR: Tumor volume reduction ratio; Peri-T: peritumor; ICIs: immune checkpoint inhibitors; ICD: immunogenic cell death; DAMPs: death-associated molecular patterns; CRT: calreticulin; ATP: adenosine triphosphate; HMGB1: High-mobility-group Box 1; DCs: Dendritic cells; HMC: major histocompatibility

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publications

All Authors have agreed to submit it in its current form for consideration for publication in your journal.

Competing interests

There are no conflicts to declare.

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Authors' contributions

Experiments were designed by Byung-Yoon Kang and conducted by Byung-Yoon Kang, Sung Min Kim, Wonhee Hur, Pu Reun Roh, and Long Jin. Data were analyzed by Byung-Yoon Kang. Ji Won Han, Pil Soo Sung, Eungyoung Tak, and Won Jong Kim offered crucial advice and examined the manuscript on every step of writing. The manuscript was prepared by Byung-Yoon Kang and edited by Ho Jong Chun and Seung Kew Yoon. All authors read and approved the final manuscript.

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- ## Figures

(A)

Figure 1

Establishment of the rat liver cancer model (A) Schematic diagram of the experimental schedule. (B) Serum ALT activity. (C) Serum creatinine value. (D) Serum AFP value. (E) MRI of liver cancer showing the size (indicated with a dotted line). (F) Image of liver cancer. (G) Histological section stained with H&E.

Figure. 2

Figure 2

TACE procedure (A) Representative TACE procedure image 1. Common carotid artery isolation 2. Common carotid artery cannulation 3. Insertion of a microcatheter into the liver 4. Drug infusion through the microcatheter (B) Angiographic images of transarterial chemoembolization in a rat liver cancer model. 1. Common hepatic arteriography (ventral view) 2. Common hepatic arteriography (lateral view) demonstrates the proper hepatic artery (arrow) and gastroduodenal artery (arrowhead). 3. Hypervascular tumor staining of the liver tumor (arrowheads)

Figure. 3

Figure 3

Anticancer effect of pPBA-Dox nanocomplexes after TACE in a rat liver cancer model (A) MRI of liver cancer showing the size in each group (indicated with a dotted line and an arrowhead). (B) Bar graph of the tumor volume in each group. (C) Volume reduction ratio (VRR) in each group. (D) AFP reduction ratio in each group. (E) Serum ALT activity in each group. (F) Serum creatinine level in each group. (G) H&E

staining in each group. The tumor necrosis area (blackstar), immune cell (arrowhead), and tumor region (dotted line).

Figure. 4

Figure 4

Immune cell changes after TACE in a rat liver cancer model (A) Strategy of FACS. (B) Number of T cells in the liver per 1 mg in each group. (C) Number of CD69⁺ T cells in the liver per 1 mg in each group. (D)

Number of Ki-67+ T cells in the liver per 1 mg in each group. (E) Number of PD-1+ T cells in the liver per 1 mg in each group. (F) Cytotoxicity assay of McA-RH7777 PBMCs and hepatic immune cells.

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