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Selective in vitro and in vivo growth inhibition against human yolk sac tumor cell lines by purified antibody against human α-fetoprotein conjugated with mitomycin C via human serum albumin

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Summary. The anticancer drug mitomycin C (MMC) was conjugated with an affinity-purified horse antibody to human α-fetoprotein (aAFP) with human serum albumin (HSA) as the intermediate drug carrier. The conjugate (aAFP:HSA:MMC molar ratio, 1:1:30) retained full antibody binding activity as determined by a competitive binding radioimmunoassay. In a cytotoxicity test in which the AFP-producing human yolk sac tumor TG-1 cells were preincubated with test materials for 2 h followed by an additional 48-h culture in fresh medium, the conjugate was 20-fold more cytotoxic than free MMC at an equivalent MMC concentration of 100 ng/ml. The in vivo antitumor effect of the conjugate was tested against the human yolk sac tumor JOG-9 growing in athymic nude mice. When the tumor-bearing mice were treated with a total of 6 injections given on 2 consecutive days and then every other day starting 8 days after SC tumor inoculation [2 (equivalent MMC) μg/head per injection], the conjugate retarded tumor growth more effectively than free MMC and normal horse immunoglobulin conjugate.

Introduction

The cytotoxic effect of drugs on the normal host cells is very frequently the cause of dose-limiting adverse effects in cancer chemotherapy, and one possible approach to the alleviation of this problem is the use of the drugs as conjugates with antibodies directed to the tumor-associated antigens on the target tumor cells [1, 6, 18].

Recently we developed a novel method of conjugation of mitomycin C (MMC) with antibody using human serum albumin (HSA) as the intermediate drug carrier [20]. This method has several features which include the following: MMC is linked to HSA by a "soft" bond which allows release of the MMC molecule for exhibition of the cytotoxic action, and the use of HSA increases the moles of the drug which can be carried per mole of the antibody with minimal damage to the solubility and the antibody binding activity.

In the present study we have prepared a HSA-mediated conjugate of MMC with an affinity-purified horse anti-human α-fetoprotein (AFP) antibody (aAFP), aAFP-HSA-MMC, and evaluated its in vivo antitumor activity against the human ovarian yolk sac tumor JOG-9 xenograft in athymic nude mice as well as its in vitro activity against the in vitro culture cell line TG-1 also originating from a human testicular yolk sac tumor. JOG-9 and TG-1 were established in our laboratories a few years ago and are AFP-producing [11]. Localization of AFP at JOG-9 implanted and grown in nude mice has previously been demonstrated in our laboratory [11].

The therapeutic effect of the aAFP-drug conjugates shown in this paper warrants further exploitation of the potential utility of the aAFP conjugates in the treatment of patients with yolk sac tumors, particularly recurrent ones frequently difficult to control with current drugs and modalities. The present study also confirms the potential utility of aAFP-drug conjugates as the cytotoxic agents selective against AFP-producing tumors indicated by previous studies [7, 16–19].

Materials and methods

Tumor cells. The human yolk sac tumor JOG-9 used in this study was established by SC inoculation of fresh tumor tissues from a 14-year-old female patient diagnosed as pure ovarian yolk sac tumor at surgery into nude mice in our laboratory [11]. The donor patient had not received chemotherapy. For serial transplantation, the tumor was cut into small pieces in ice-cold Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS) under sterile conditions and transplanted SC into nude mice.

Histologic appearance of the serially transplanted tumor was identical to that of the original tumor. The tumor used in this study was at its 17th to 20th passage in nude mice. The tumor-take rate in nude mice was about 95%.

The in vitro cell line designated as TG-1 was established as previously reported from the AFP-producing human testicular yolk sac tumor JTG-1 transplanted in nude mice [10]. To remove the contaminating mouse fibroblasts from the primary culture the method described by Okabe et al. was used [12]. The TG-1 cells grew well forming colonies and multilayers without contact inhibition in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Grand Island Biological Co., Grand Island, NY, USA) under usual culture conditions. The cells were polygonal or spherical in shape and preserved the AFP-producing ability.

Animals. Female athymic nude mice (*nu/nu*, BALB/c background) 6 to 8 weeks old were used in this study.

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These specific-pathogen-free (SPF) mice were obtained from Clea Japan Inc., Tokyo, Japan and maintained under SPF conditions using vinyl isolators.

aAFP. Horse antiserum against human AFP was produced by weekly SC injections of 1 mg of purified human AFP emulsified in Freund's complete adjuvant. Highly purified antibody to the AFP was obtained using affinity chromatography as previously reported [5].

Preparation of conjugates. The HSA-mediated conjugate of MMC with aAFP, aAFP-HSA-MMC, was prepared according to the procedure previously reported [20]. MMC was linked to HSA at the aziridine nitrogen atom through a glutaric acid-derived spacer arm. HSA-MMC was then conjugated mainly via the single free thiol group of HSA with aAFP having the maleimide group introduced. The average molar binding ratio of aAFP:HSA:MMC was 1:1:30 as determined by the methods previously described [20]. Using normal horse immunoglobulin (nIg) a similar conjugate, nIg-HSA-MMC, was prepared.

Determination of antibody binding activity of the conjugate. A 50 µl sample of ^{125}I-labeled human AFP (sp. act. 100 mCi/mg protein) diluted with 10% normal horse serum in PBS was mixed with 100 µl of either aAFP or aAFP-HSA-MMC ($0.1-10^4$ µg specific antibody/ml) in microtitration plates (Costar No. 2595; Costar, Cambridge, Mass., USA) and incubated for 2 h at room temperature on a horizontal shaker. Then paper discs with which aAFP was covalently coupled (10 µg/disc) were put into the wells as the solid phase immunoadsorbent, and the mixtures were reincubated for 18 h under the same conditions as the first incubation. The discs in the wells were rinsed three times with 0.2 ml of PBS, and transferred to tubes for counting (Autologic gamma-counter, Dinabot, Tokyo, Japan). The results were expressed by percent binding of ^{125}I AFP as calculated by the following equation: % binding = (cpm on disc incubated with test material – cpm on disc incubated with PBS containing 10% horse serum)/total cpm added × 100.

Cytotoxic test. TG-1 cells were harvested with 0.02% EDTA-containing PBS, washed throughly with PBS, resuspended in RPMI 1640 medium supplemented with 15% FCS, and used in two forms of cytotoxicity tests.

(1) The 48-h cytotoxicity test: 1×10^5 cells in 0.5 ml of growth medium per well were plated out in 24-well microtiter plates (Corning No. 25820, Corning Glass Works, Corning, NY, USA) and incubated for 2 h to allow the cells to become adherent. Various dilutions in 0.5 ml of growth medium of the drug or conjugate which had been filtrated through 0.22 µm Millex filter (Millipore, Bedford, Mass., USA) were added to each well. The plates were incubated for 48 h at 37 °C.

(2) The 2-h pretreatment cytotoxicity test: aliquots of 1×10^6 cells in growth medium were spun down in plastic centrifuge tubes (Corning No. 25330). The pellet was resuspended in 0.5 ml of various dilutions of the drug or conjugate in growth medium, incubated for 2 h at 37 °C, and centrifuged. The supernatant was removed, and the cells were washed twice in 15 ml of growth medium followed by final resuspension in growth medium. Aliquots of the medium containing 1×10^5 cells were plated in 24-well microplates, and the plates were cultured at 37 °C for 48 h.

After 48-h incubation 10 µCi of ^{3}H thymidine (sp. act. 20 Ci/mmol; New England Nuclear, Cambridge, Mass., USA) was added to each well, and the plates were incubated for a further 2 h. Then 1 ml of 10% trichloroacetic acid (TCA, Sigma, St. Louis, Mo., USA) was added to each well and the plates were kept at 4 °C overnight. The TCA precipitates were washed in 5% TCA, solubilized in 0.5 ml of 1 *N* NaOH, transferred to vials, and mixed with Triton X-100 scintillator for assay in a liquid scintillation counter (Aloka LSC900 liquid scintillation counter, Tokyo, Japan).

Therapeutic experiments. Nude mice were inoculated with a 2-mm peice of JOG-9 SC in the back. On day 8 after tumor inoculation when the tumor volume as calculated by the equation below had become about 18 mm^3, the mice were divided into an appropriate number of groups with each group consisting of 5 mice, and treatment was initiated. The test materials [2 (equivalent MMC) µg/mouse] in 0.1 ml PBS were injected IP into the tumor-bearing nude mice either every 5 days (experiment A) or on 2 consecutive days and then every other day (experiment B) for a total of 6 doses. Control mice were given the same volume of PBS.

Evaluation of antitumor effect. The size of the growing transplanted tumors was measured with a sliding caliper every 5 days, and the volume (V) was calculated using the formula $V\ (mm^3) = L \times W^2 \times ½$, where L and W are the length (long axis) and width (short axis) in millimeters, respectively. For comparison among different groups, the mean relative tumor volume (RV) for each group was calculated from the formula $RV = V_i/V_o$, where V_i is the mean tumor volume at any given time and V_o is the mean initial tumor volume when the treatment began, because individual tumor growth showed variation. The ratio of RV of the treated group to RV of the control group (T/C) was calculated. At the end of the treatment studies, all treated and control mice were sacrified. The tumors were resected and weighed, and T/C in weight was calculated [15].

Serum AFP determination. Serum AFP levels of tumor-bearing nude mice were measured by a sandwich radioimmunoassay at regular intervals using blood obtained from a tail vein [16].

Toxicity of test materials. As a monitoring of the side effects of the test materials, the body weight of the mice was measured once a week during the experimental period.

Statistical analysis. The results were analyzed using the Student's *t*-test.

Results

Antibody activity of the conjugate

The antigen-binding activity of aAFP and aAFP-HSA-MMC was measured by a competitive binding radioimmunoassay. No original antibody activity was reduced after MMC was bound to the antibody through HSA as the intermediate drug carrier (Fig. 1).

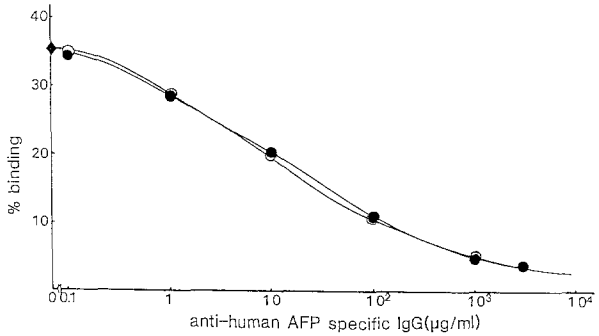

Fig. 1. Antibody binding activity of aAFP-HSA-MMC. The antibody binding activity of aAFP (○) and of aAFP-HSA-MMC (●) was measured by competitive binding radioimmunoassay. ^{125}I-labeled human AFP was mixed with either aAFP or the conjugate in microtitration plates and incubated for 2 h at room temperature. Then aAFP-coated discs were put into the wells and the plates were reincubated for 18 h at room temperature. The discs were washed with PBS and counted in a gamma counter. The results are expressed as percent binding of ^{125}I AFP. Points represent means of triplicate determinations. SD for each point was smaller than the point as plotted. The percent binding observed when neither aAFP nor aAFP-HSA-MMC was added is shown by ◆

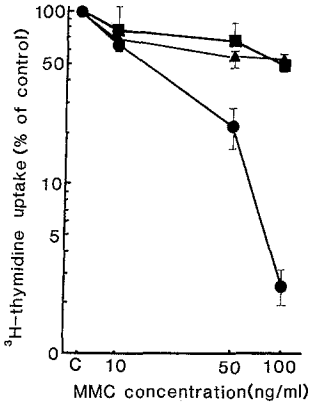

Fig. 3. Specific cytotoxicity of aAFP-HSA-MMC. The cytotoxic effect of free MMC (▲), nIg-HSA-MMC (■), or aAFP-HSA-MMC (●) on TG-1 cells was assayed by the 2-h pretreatment test in terms of percent uptake of ^{3}H thymidine as compared with that of control. For detail, see *"Materials and methods"*. Point, mean of triplicate determinations; bar, SE (indicated unless smaller than the point as plotted)

Cytotoxic activity of aAFP-HSA-MMC to tumor cells in vitro

The cytotoxicity of aAFP-HSA-MMC against the yolk sac tumor cell line TG-1 was assessed by determination of the inhibition of the ^{3}H thymidine uptake by the cells that were cultured with the conjugate added to the medium (Fig. 2). A cytotoxicity test in which target cells were continuously incubated with the test materials for 48 h was designed to assess the retention of cytotoxic activity in the conjugate. The aAFP conjugate exhibited a potent concentration-dependent inhibitory effect at concentrations above 10 ng (equivalent MMC)/ml. The inhibitory effect of the conjugate was about the same degree as that of unconjugated MMC. These results indicate that the potent cytotoxicity of MMC was almost completely preserved after the conjugation procedure. To study the selective cytotoxicity of the conjugate under conditions that somewhat mirror the in vivo situation, the cells were incubated with the conjugate or other test materials (fee MMC or nIg-HSA-MMC) for 2 h before the unbound material was removed by washing followed by an additional 48-h culture of the cells in fresh growth medium. This was based on our observation that aAFP binding to TG-1 cells reached a plateau level within 2 h at 37 °C (data not shown). As shown in Fig. 3, under these conditions the cytotoxicity of MMC was enhanced by conjugation with aAFP. The conjugate showed about 20-fold greater cytotoxicity than free MMC at an equivalent MMC concentration of 100 ng/ml. A significant difference in the cytotoxicity between aAFP-HSA-MMC and nIg-HSA-MMC was also observed. The cytotoxicity of nIg-HSA-MMC was similar to that of free MMC at equivalent MMC concentrations of 50 and 100 ng/ml. Previous work in this laboratory has shown that aAFP alone has no effect on the thymidine uptake by TG-1 cells at concentrations (0.75–1.5 μg/ml) higher than the concentration (amount) of antibody in the conjugate.

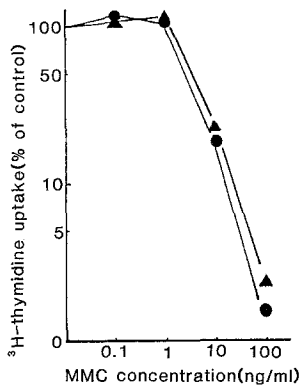

Fig. 2. Retention of drug cytotoxic activity in aAFP-HSA-MMC. The cytotoxic effect of free MMC (▲) and of conjugate (●) on AFP-producing TG-1 cells was assayed by the 48-h cytotoxic test in terms of percent ^{3}H thymidine uptake as compared with that of control. For detail, see *"Materials and methods"*. Point, mean of duplicate determinations

Therapeutic effect of aAFP-HSA-MMC conjugate

The in vivo antitumor activity of the aAFP-HSA-MMC conjugate against the AFP-producing human yolk sac tumor JOG-9 transplanted in nude mice was examined. The experimental data obtained are shown in Fig. 4, the results being presented as mean relative tumor volumes both in treated and in control groups.

In experiment A, nIg-HSA-MMC had only a moderate inhibitory effect on tumor growth at day 35 (T/C ± SE, 65 ± 10%), whereas both MMC and aAFP-HSA-MMC suppressed the growth of JOG-9 significantly. Although a slight retardation of the tumor growth was observed with aAFP-HSA-MMC in comparison with MMC up to 16 days after tumor inoculation, the rate of suppression by aAFP-HSA-MMC (T/C, 33 ± 6%) was similar to that of free MMC (T/C, 30 ± 9%) at the final stage of this treatment study.

In experiment B, the tumor initially grew at a lower rate in the mice treated with aAFP-HSA-MMC or with free MMC than that in mice treated with nIg-HSA-MMC

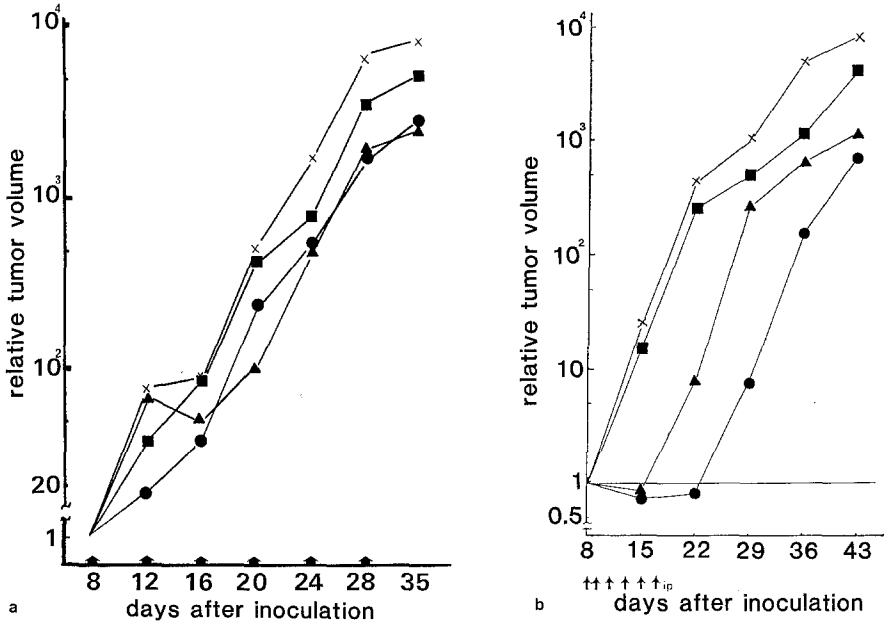

Fig. 4. In vivo antitumor activity of aAFP-HSA-MMC. Groups of 5 athymic nude mice were inoculated SC with the human ovarian yolk sac tumor JOG-9 (day 0). Different groups received an IP injection of 2 (equivalent MMC) mg/head of free MMC (▲), nIg-HSA-MMC (■), or aAFP-HSA-MMC (●), or PBS (×) either every 5 days **(a)** or on 2 consecutive days and then every other day **(b)** for a total of 6 doses starting on day 8 as indicated by arrows. Results are expressed as RV for each group

or just PBS. Although at 29 days after tumor inoculation in the specific conjugate group and at 22 days in the MMC group (11 and 4 days after treatment cessation, respectively) the tumor growth rate had become similar to that of the PBS group, the difference in tumor volume between the control PBS or nIg-HSA-MMC group and the MMC or aAFP-HSA-MMC group was statistically highly significant ($P<0.025$) during the initial phase of retardation (up to 22 days after inoculation). The aAFP-HSA-MMC conjugate-treated group showed a significant suppression of tumor growth at day 43 (T/C, 9±0.9%) in comparison with that of MMC-treated mice (T/C, 26±15%) as well as other controls ($P<0.05$).

The AFP levels in the sera paralleled the tumor volumes of each group (Fig. 5). High and moderate AFP levels were observed in the groups of mice treated with just PBS or nIg-HSA-MMC, and with free MMC, respectively. A low AFP level was maintained for a considerably long period of time in the group of mice injected with aAFP-HSA-MMC, although the amount of aAFP in the conjugate was too small to neutralize the serum AFP in the tumor-bearing nude mice by aAFP alone.

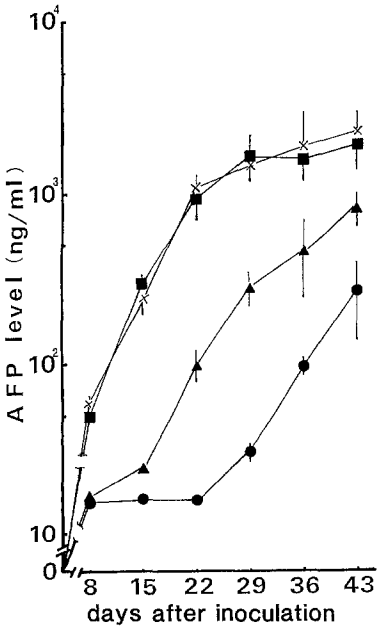

Fig. 5. Serum AFP level in nude mice treated with test materials in experiment B. The serum AFP level was determined by radioimmunoassay with respect to mice treated with PBS (×), MMC (▲), nIg-HSA-MMC (■), or aAFP-HSA-MMC (●). Point, mean; bar, SD (indicated unless smaller than the point as plotted)

Toxicity of the test materials

No significant weight loss was found in any group of mice (data not shown).

Discussion

For conjugation of MMC with aAFP, we used HSA as the intermediate drug carrier. In this method of conjugation MMC is first linked to HSA, and HSA-MMC is coupled with aAFP [20]. This is to avoid a detrimental effect on the antibody binding activity with a high degree of drug binding. Indeed, the aAFP activity in the conjugate to bind to AFP was identical to the free aAFP binding activity as determined by competitive radioimmunoassay using ^{125}I-labeled AFP. In the 48-h cytotoxicity test in which the test samples were allowed contact with the cells throughout the 48-h culture period, aAFP-HSA-MMC was as cytotoxic as unconjugated MMC. In aAFP-HSA-MMC, the drug is linked to HSA with the cleavable aziridyl amide bond [20] that should contribute to the retention of the drug activity after conjugation. In conclusion, the results obtained indi-

cate that the conjugate retains both antigen-binding activity and drug cytotoxicity. Several investigators have reported partial inactivation of drug cytotoxicity and antibody binding activity after conjugation [1, 3, 4, 6, 13].

The selective cytotoxicity of aAFP-HSA-MMC was assessed by the 2-h preincubation test in which the test materials were allowed contact with the cells only for the first 2-h period. This in vitro test is supposed to be somewhat similar to in vivo conditions as compared with the 48-h cytotoxicity test. Similar evaluation protocols for the cytotoxicity of the conjugates have also been used by other investigators [3, 4]. In the preincubation test the aAFP specific conjugate showed a much higher cytotoxicity than did the nIg conjugate. Moreover, MMC activity was enhanced by conjugation with aAFP. The former observation strongly suggests the involvement of specific antibody/antigen interaction in the cytotoxicity of aAFP-HSA-MMC.

The in vivo antitumor effect of aAFP-HSA-MMC against the human yolk sac tumor JOG-9 xenografted in athymic nude mice was assessed by treating the mice with a total of 6 doses given either every 5 days (experiment A) or on 2 consecutive days and then every other day (experiment B) both starting 8 days after tumor inoculation. The aAFP conjugate showed tumor growth inhibitory effect on either of the two schedules. The conjugate was more efficacious than the corresponding nIg conjugate again supporting the concept that the aAFP conjugate is not acting just as a mere nonspecific high molecular weight drug but acting with the specific antigen-antibody reaction being involved in the mechanism of action.

In experiments A and B, in which the same single and total dosage was employed, administration of the conjugate at shorter intervals within a shorter period after the tumor inoculation (experiment B) was more effective in suppression of the tumor growth. In this protocol of intensive treatment at an early stage of tumor, unconjugated MMC and aAFP-HSA-MMC completely suppressed the tumor growth equally at least during the administration period. However, a longer retardation of the onset of tumor growth was brought about by the conjugate, leading to a greater suppression of the tumor volume at the final day of the observation in mice treated with the conjugate rather than with free MMC. Various factors such as the tumor size and the serum AFP level at the time of the conjugate injection and the serum half-life of the xenogeneic immunoglobulin in mice play a role in determining the efficacy of the aAFP conjugate. Further detailed investigations and optimization of the treatment protocol will be the next step of our studies on the aAFP conjugate.

The serum AFP levels of the respective groups of mice in experiment B paralleled the tumor size. This supports the effectiveness of the conjugate and also indicates that the tumor cells surviving the aAFP conjugate treatment were producing as much AFP as the original tumor cells. No selection of low AFP producers seemed to have occurred through the treatment with the aAFP conjugate. The JOG-9 cells all may have been producing and expressing sufficient AFP for the cells to be killed by the aAFP conjugate. Alternatively MMC released from the conjugate extracellularly in the tumor tissue may have exerted cytocidal action on the low AFP producer cells. This mode of action is an alternative to endocytic entry of the conjugate into the cell followed by the intracellular release of active MMC molecules.

In the present investigation in our series on the drug conjugates of aAFP, we studied the in vitro and in vivo antitumor activities of aAFP-HSA-MMC against yolk sac tumors. Yolk sac tumors are highly malignant germ cell tumors of the ovary, testis, and other sites in children or young adults [8, 9, 10, 14]. The prognosis of patients with these tumors has been improved by the progress of cancer chemotherapy as well as surgery [2, 8]. However, recurrent ovarian yolk sac tumors are still often fatal and such a situation occurs from subclinical micrometastasis [8, 14]. The results presented in this paper form a basis for the exploitation of the potential therapeutic utility of the aAFP-HSA-MMC conjugate against AFP-producing yolk sac tumors. A potential application of the conjugate to further improve the prognosis of patients with yolk sac tumor might be intra-abdominal administration of the conjugate after surgical removal of the primary tumors as the adjuvant chemotherapy to eliminate subclinical metastasis.

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