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科学研究費助成事業　　研究成果報告書

令和　５ 年　 ６ 月 １４ 日現在

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研究課題名（和文）Development of clinical-fit aptamer targeting CYP24A1 using an integrated approach of high-speed atomic force microscopy and molecular docking

研究課題名（英文）Development of clinical-fit aptamer targeting CYP24A1 using an integrated approach of high-speed atomic force microscopy and molecular docking

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交付決定額（研究期間全体）：（直接経費）　　3,600,000円

研究成果の概要（和文）：in vivo 研究により Apt-7 を評価しました。 in vivo でのヌクレアーゼ耐性を強化するために、Cb-7 と呼ばれる Apt-7 の環状二価バージョンを設計および開発し、 Cb-7 は、肺癌細胞株 A549 細胞において、機能活性の増加と顕著な抗増殖効果を伴う高いヌクレアーゼ安定性を示しました。 予備試験として、in vivo で Cb-7 を調査しました。 A549細胞を用いた異種移植マウスモデルの評価系を確立しました。 1,25D3、Cb-7 は週 3 回、左肩の腫瘍のみに直接投与されました。 その結果、Cb-7あり の腫瘍体積は減少傾向を示しました。

研究成果の学術的意義や社会的意義
The novel aptamer identified in this study presents an opportunity to generate a new probe for the recognition and inhibition of CYP24 for biomedical research and could assist in the diagnosis and treatment of cancer.

研究成果の概要（英文）：Aptamer Apt-7 has shown high binding affinity, specificity, and inhibitory potency against CYP24 as well as anti-proliferative ability in vitro and in cellular assays. Next, we evaluated Apt-7 by in vivo study. To enhance the nucleases resistance in vivo, we designed and developed a circular bivalent version of Apt-7, called Cb-7, and performed systematic studies of Cb-7. Cb-7 showed high nuclease stability with increased functional activity and remarkable anti-proliferative effects in lung cancer cell line A549 cells. As a preliminary test, we investigated Cb-7 in vivo. We established an evaluation system of a xenograft mouse model with A549 cells. 1,25D3 was administered intraperitoneally three times a week, while Cb-7 was administrated directly into the tumor three times a week only in the tumor of the left shoulder. As the result, the tumor volume of the left shoulder (with Cb-7) showed a tendency to decrease. However, further study is needed in the future.

研究分野：Life Sciences

キーワード：CYP24　Vitamin D3　Aptamer　in vivo　A549 cells

１．研究開始当初の背景(Introduction)

Vitamin D3 is metabolized to 1α, 25-dihydroxy vitamin D3 (1,25D3), which exerts various physiological effects such as bone formation, cell proliferation, and differentiation. Thus, 1,25D3 and its analogs have been clinically used as therapeutic agents against rickets and psoriasis, furthermore, they have been also expected as cancer treatments. CYP24A1 (CYP24) is a key enzyme that catalytically degrades the 1,25-D3, thus overexpression of CYP24 leads the various types of diseases such as osteoporosis and cancer. Over the past decade, several types of CYP24-resistant 1,25D3 analogs have been extensively synthesized and developed as anti-cancer agents for in vitro and in vivo studies. However, side effects and the development of drug resistance have limited their successful outcomes in clinical trials so far.

We recently identified CYP24-inhibiting DNA aptamer (Apt-7), as an alternative approach, which enhances vitamin D3 functionality in cancer cells. The next challenge to proceed into in vivo study is overcoming the issues such as low stabilization of the labile nature of DNA aptamers against nucleases and limited distribution to targeted tumor tissue.

２．研究の目的(Aim of research)

The purpose of this study is to optimize and stabilize the Apt-7 and to evaluate the biological effects of the advanced Apt-7 by in vivo study. First, Apt-7 will be shortened and engineered to improve the stability, affinity, and inhibition efficacy. Then, we will demonstrate the significance of the advanced Apt-7 using tumor-bearing mice. Through this study, we ultimately aim to make progress toward the application of Apt-7 for cancer therapy.

３．研究の方法(Experimental methods)

1. Construction of circular bivalent version of Apt-7 (Cb-7)

To prepare Cb-7 aptamer, its two components were first dissolved in T4 DNA Ligase buffer in appropriate concentration and then heated at 95 °C for 5 min, followed by quick chilling to 16 °C, allowing the formation of circular aptamer with two nicks. The nick aptamer was then incubated with T4 DNA ligase at 16 °C for 12 h to form Cb-7. Finally, the solution was incubated at 75 °C for 10 min to denature the ligase. DNA samples were extracted by phenol-chloroform extraction and ethanol precipitation. The concentration of DNA was determined with UV-vis spectrophotometry prior to usage.

2. Tumor growth inhibition

A total of $5x10^6$ cells per mouse were implanted into the right shoulder of nude mice (BALB/c-nu/nu, 4 weeks old, female), and tumor volumes were measured over time. The tumor volume reached approximately 100 mm^3, 1,25D3 was administered intraperitoneally three times a week at a dose of 0.3 μg/kg-bw, while Cb-7 was administrated directly into the tumor three times a week at a dose of 100 μg/kg only in the tumor of the left shoulder. At the end of the treatment, the tumor's major diameter and minor diameter were measured, and the tumor volume was calculated.

４．研究成果(Results)

In our previous studies, aptamer Apt-7 has shown high binding affinity, specificity, and inhibitory potency against CYP24 as well as anti-proliferative ability in vitro and in cellular assays. Next, we sought to evaluate Apt-7 by in vivo study. However, aptamers are generally susceptible to plasma exonucleases in vivo. This seriously affects their in vivo applications. To enhance the nucleases resistance in vivo, we designed and developed a circular bivalent version of Apt-7, called Cb-7, and performed systematic studies of Cb-7 to examine the nuclease stability, functional activity, and anti-cancer effects in lung cancer cell line A549 cells. As a preliminary test, we investigated Cb-7 in vivo.

1. Design and synthesis of circular bivalent Apt-7 (Cb-7)

A shorter sequence has merits so that it can reduce the molecular size and it makes more available for synthesis and drug delivery. Therefore, we removed some nucleotides that are not essential for CYP24 binding. Furthermore, the nuclease stability of aptamer is a fundamental requirement for successful application in vivo. Many studies demonstrated that circular bivalent aptamers have higher nuclease resistance than monomers (Hailan Kuai et al., J. Am. Chem. Soc. 2017). Therefore, we constructed a circular bivalent aptamer-7 (Cb-7) for improving the nuclease stability of Apt-7 (Fig. 1). Then, we examined the stability of Cb-7 in 95% fetal bovine serum (FBS) at different time intervals (0 h, 1 h, and 4 h). The results were analyzed by agarose gel electrophoresis.

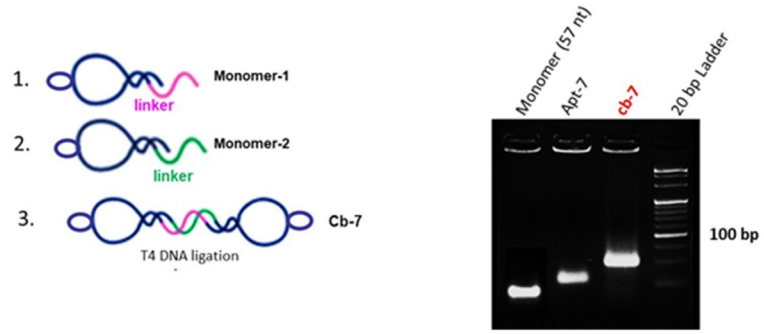


Fig. 1. Design and synthesis of circular bivalent aptamer Apt-7 (Cb-7).

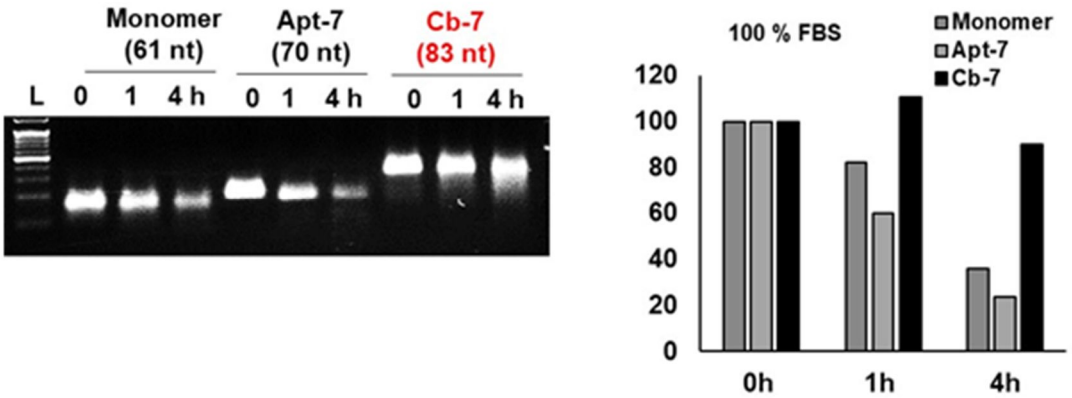


Fig. 2. Nuclease stability of Cb-7 in serum.

As the result of electrophoresis, Cb-7 showed an intact band while monomer and Apt-7 showed rapid degradation (Fig. 2). Our aptamer stability assay suggests that Cb-7 has acquired sufficient stability in serum and can be utilized for in vivo studies.

2. Evaluation of in vitro CYP24 inhibition efficacy of Cb-7

Next, we investigated whether Cb-7 maintains the ability as Apt-7 to inhibit CYP24 in vitro. We evaluated Cb-7 inhibitory potency by examining E. coli expressing CYP24 activity in a reconstitution system containing membrane fraction prepared, with its electron transfer system. Fig. 3 shows the inhibitory effects of Cb-7 on CYP24 activity at different concentrations. The HPLC analysis results showed that Cb-7 reduced the activity of CYP24 in a dose-dependent manner with improved inhibitory effects compared to Apt-7.

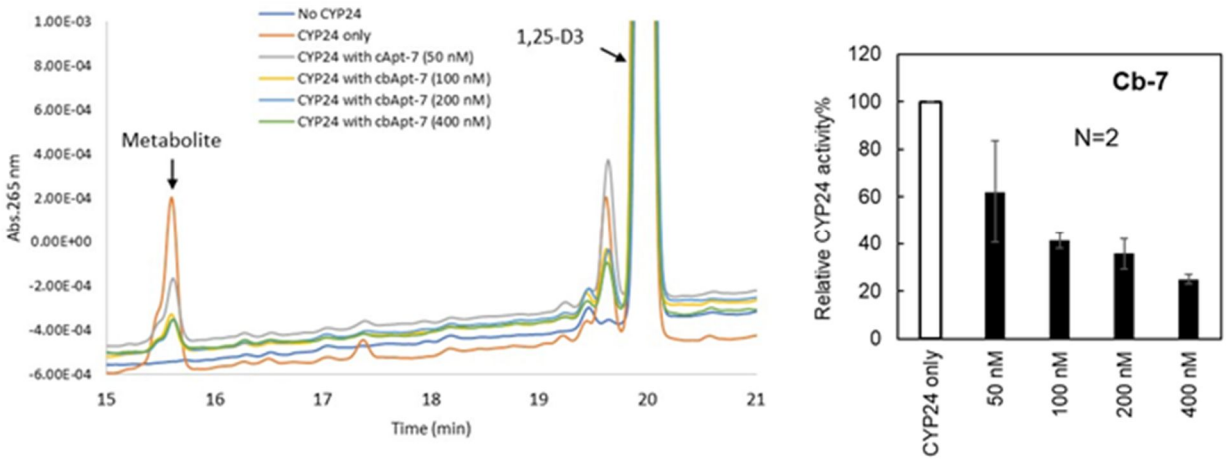


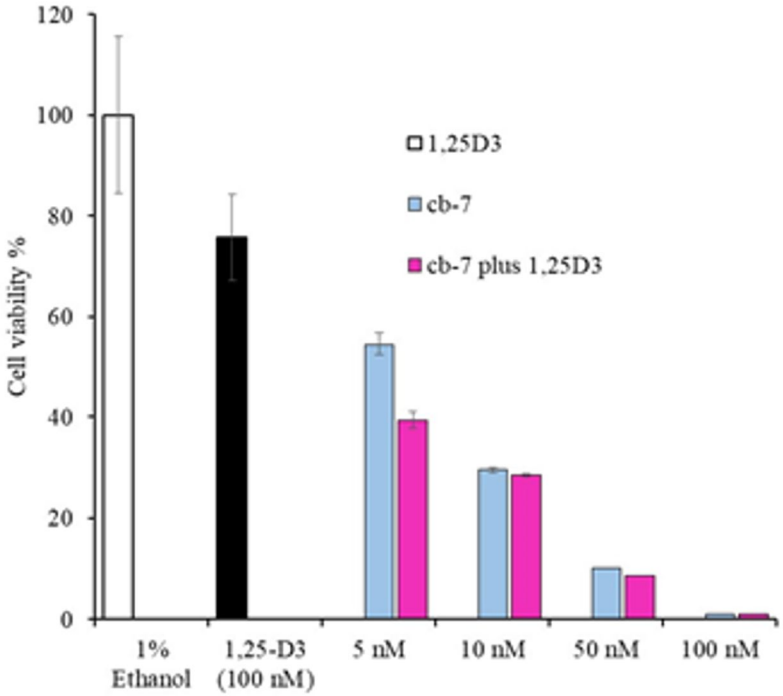
Fig. 3. In vitro CYP24-inhibition efficacy of Cb-7.

3. Effect of Cb-7 on cell viability of A549 cells

Following the inhibition of CYP24 by Cb-7, we then examined the effects of Cb-7 on the antiproliferative action of 1, 25-D3 in A549 cancer cells. To study the inhibitory effects of Cb-7 on enhanced 1,25-D3 antiproliferative activity, the cells were cultured in the presence or absence

of Cb-7 (0, 5, 10, 50, and 100 nM). Interestingly, the inhibition of A549 cell proliferation was observed after 48 h of aptamer treatment (Fig. 4). The results reveal that cell viability of A549 cells was remarkably decreased with treatment of 100 nM 1,25-D3 together with increasing concentration of Cb-7.

Fig. 4. Effect of Cb-7 on cell viability of A549 cells.



4. Effects of 1, 25-D3 on tumor-bearing mice with and without treatment of Cb-7

As a preliminary step to investigate the in vivo effects of Cb-7, we first established an evaluation system of a xenograft mouse model with A549 cells. A total of 5x106 cells per mouse were implanted into the right shoulder of nude mice (BALB/c-nu/nu, 4 weeks old, female), and tumor volumes were measured over time. The tumor volume reached approximately 100 mm3, the average volume at the start of general treatment, after about 2-3 weeks of cell transplantation, and continued to increase thereafter. As we confirmed the generation of tumor-bearing mice with A549 cells, we next considered the dosage and administration method of 1,25D3. When 1,25D3 was directly administered into the tumor at a dose of 0.3 or 0.6 ng/kg-bw twice a week, no significant difference was observed in either group compared to the non-treatment group. Some cells were necrotic in the 1,25D3-administered group, so there may have been an effect on the cells near the injection site. But it was speculated that 1,25D3 did not reach the entire tumor cells and did not lead to a decrease in tumor volume. Initially, we considered administering 1,25D3 and Cb-7 together directly into the tumor, but based on these results, we decided to administrate 1,25D3 by intraperitoneal injection and Cb-7 by intratumoral injection.

Next, A549 cells were implanted into both the left and right shoulders of nude mice. After tumor volume reached 100 mm2, 1,25D3 was administered intraperitoneally three times a week at a dose of 0.3 μg/kg-bw, while Cb-7 was administrated directly into the tumor three times a week at a dose of 100 μg/kg only in the tumor of the left shoulder. As the results, the tumor volume of the right shoulder (without Cb-7) showed similar to that of the untreated group, while the tumor volume of the left shoulder (with Cb-7) showed a tendency to decrease, its tumor volume was similar to that of 1,25D3 alone at 3 μg/kg-bw, 10 times the above dose. However, there were no significant differences among each group, so a more detailed analysis is required in the future.

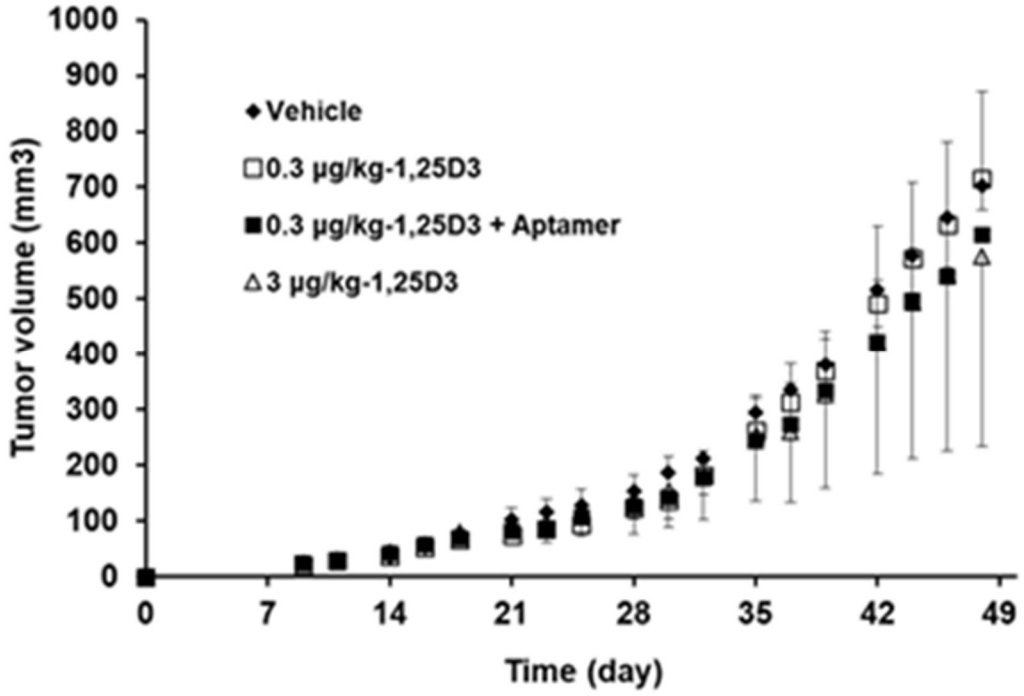


Fig. 5. The tumor volume of tumor-bearing mice with or without treatment.
□: No treatment
□: 0.3 μg/kg-b.w of 1,25D3
■: 0.3 μg/kg-b.w of 1,25D3 + 100 μg/kg-b.w of Cb-7
△: 3 μg/kg-b.w of 1,25D3
1% ethanol with (□, ■, and △) or without (□)1,25D3 containing PBS were intraperitoneally administrated, and Cb-7 (■) was administrated directly into the tumor. Each point indicates the average of each group (N=3)

５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　0件）

１．著者名 Madhu Biyani, Kaori Yasuda, Yasuhiro Isogai, Yuki Okamoto, Wei Weilin, Noriyuki Kodera, Holger Flechsig, Toshiyuki Sakaki, Miki Nakajima, and Manish Biyani	４．巻 14, 16
２．論文標題 Novel DNA Aptamer for CYP24A1 Inhibition with Enhanced Antiproliferative Activity in Cancer Cells	５．発行年 2022年
３．雑誌名 ACS Applied Materials & Interfaces	６．最初と最後の頁 18064-18078
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/acsami.1c22965	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

〔学会発表〕　計6件（うち招待講演　4件／うち国際学会　3件）

１．発表者名 Madhu Biyani
２．発表標題 Study of the binding mechanism of aptamer to CYP24A1 by an integrated approach of HS-AFM and molecular docking
３．学会等名 The 36th JSSX Annual Meeting (2021)（招待講演）
４．発表年 2021年

１．発表者名 Madhu Biyani
２．発表標題 In vitro selection of a DNA aptamer inhibiting human CYP24A1
３．学会等名 The 94th Japanese Biochemical Society (2021)
４．発表年 2021年

１．発表者名 Madhu Biyani
２．発表標題 Integration of a high-speed atomic force microscopy and a computational microscopy: from selection hits to clinical leads
３．学会等名 The 16th Anniversary India-Japan Fest, BICON2021, Virtual International Conference, Dec. 14-18, 2021（招待講演）（国際学会）
４．発表年 2021年

１．発表者名
Madhu Biyani

２．発表標題
APPLICATIONS OF APTAMERS AS RESEARCH TOOLS: PROBLEMS AND SOLUTIONS

３．学会等名
AICTE Training And Learning Academy (ATAL), Online Faculty Development Program (FDP) 2021（招待講演）（国際学会）

４．発表年
2021年

１．発表者名
Madhu Biyani

２．発表標題
Binding properties of aptamer to CYP24, a target of cancer therapy, using an integrated approach of high-speed AFM and molecular docking

３．学会等名
6th NanoLSI Symposium, Nanoprobe Technology for Understanding Molecular Systems, November 14-15, 2022, Kanazawa, Japan.

４．発表年
2022年

１．発表者名
Madhu Biyani

２．発表標題
A novel DNA aptamer for CYP24 inhibition exerts a therapeutic effect by enhancing the anti-proliferative function of vitamin D3 in lung cancer cells

３．学会等名
WORKSHOP ON VITAMIN D, Sep 6, 2022, Austin, Texas, USA（招待講演）（国際学会）

４．発表年
2022年

〔図書〕　計0件

〔産業財産権〕

〔その他〕
ptamer Apt-7 has shown high binding affinity, specificity, and inhibitory potency against CYP24 as well as anti-proliferative ability in vitro and in cellular assays. Next, we evaluated Apt-7 by in vivo study. To enhance the nucleases resistance in vivo, we designed and developed a circular bivalent version of Apt-7, called Cb-7, and performed systematic studies of Cb-7. Cb-7 showed high nuclease stability with increased functional activity and remarkable anti-proliferative effects in lung cancer cell line A549 cells. As a preliminary test, we investigated Cb-7 in vivo. We established an evaluation system of a xenograft mouse model with A549 cells. 1,25D3 was administered intraperitoneally three times a week, while Cb-7 was administrated directly into the tumor three times a week only in the tumor of the left shoulder. As the result, the tumor volume of the left shoulder (with Cb-7) showed a tendency to decrease. However, further study is needed in the future.

６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関