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Toxicity studies of coumarin 6-encapsulated polystyrene nanospheres conjugated with peanut agglutinin and poly(N-vinylacetamide) as a colonoscopic imaging agent in rats

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Abstract

We are investigating an imaging agent that detects early-stage primary colorectal cancer on the mucosal surface in real time under colonoscopic observation. The imaging agent, which is named the nanobeacon, is fluorescent nanospheres conjugated with peanut agglutinin and poly(N-vinylacetamide). Its potential use as an imaging tool for colorectal cancer has been thoroughly validated in numerous studies. Here, toxicities of the nanobeacon were assessed in rats. The nanobeacon was prepared according to the synthetic manner which is being established as the Good Manufacturing Practice-guided production. The rat study was performed in accordance with Good Laboratory Practice regulations. No nanobeacon treatment-related toxicity was observed. The no observable adverse effect levels (NOAEL) of the nanobeacon in 7-day consecutive oral administration and single intrarectal administration were estimated to be more than 1000 mg/kg/day and 50 mg/kg/day, respectively. We concluded that the nanobeacon could be developed as a safe diagnostic agent for colonoscopy applications.

Keywords

Toxicity study; Good Laboratory Practice; Colonoscopic imaging agent; Nanosphere; Colorectal cancer

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Introduction

For the past two decades, significant progress in nano-sized biomaterials has largely contributed to medical innovation.^{1–4} Various nano-sized biomaterials have been developed and tested for biomedical applications. For instance, liposomes, which are nano-sized particles composed of lipid bilayers, are being clinically used as vehicles that improve the pharmacokinetic properties of the original drugs. An injection of liposomes encapsulating amphotericin B, which is an antifungal agent, became the first liposomal drug when it was launched in 1990.^{5,6} A conventional injection of amphotericin B was approved in 1962; however, its use was largely limited due to severe toxicity, especially for nephrotoxicity. Notably, the toxicity was dramatically reduced when amphotericin B was encapsulated into liposomes nevertheless the efficacy of the drug remained unchanged. Liposomes coated with polyethylene glycol are also used in clinics as carriers for the efficient delivery of anticancer drugs to tumor tissues based on the effect of enhanced permeability and retention.^{7–10} The potential of liposomes as safe and effective drug carriers has been validated in a large number of animal studies and clinical applications. At present, more than 10 liposomal drugs are in the market.

We have been investigating coumarin 6-encapsulated polystyrene nanospheres conjugated with peanut agglutinin (PNA) and poly(N-vinylacetamide) (PNVA), which is named the nanobeacon, as a topical probe for detection of colorectal cancer by fluorescence colonoscopy (see Supplementary materials).^{11–19} Colorectal cancer initially develops in the mucous membrane of the large intestine. In the process, the Thomsen-Friedenreich (TF) antigen is specifically expressed on the mucosal side of cancer cells.²⁰ The TF antigen is also a malignancy index for colorectal cancer.²¹ The terminal sugar is β -D-galactosyl-(1–3)-N-acetyl-D-galactosamine (Gal- β (1–3)GalNAc), which is masked by an oligosaccharide side chain extension or sialylation in normal cells.²⁰ Lectins are proteins that recognize and bind reversibly to specific carbohydrate residues expressed on the cell surface.²² PNA, which is a typical lectin that recognizes Gal- β (1–3)GalNAc, is immobilized on the nanosphere surface as a targeting moiety that binds to the TF antigen through sugar recognition. Our previous researches have revealed that PNVA rarely adheres to the intestinal mucosa.^{23–25} The nanosphere surface is modified with PNVA in order to enhance the specificity of PNA by reducing nonspecific interactions between nanospheres and normal tissues. Coumarin 6, which is a commercial product with high fluorescence quantum efficiency, is encapsulated into the nanosphere core to produce fluorescence intensity that is endoscopically detectable. Colorectal cancer can be detected through a clear fluorescence contrast between normal tissues and the nanobeacon-bound cancer tissues observed with a fluorescence endoscope. We previously reported that the nanobeacon recognized minute colorectal tumors on the mucosal surface with high affinity and specificity in orthotopic mouse models.^{12,13} The nanobeacon also detected dynamic changes of the TF antigen in human colonic tissues taken from patients with colorectal tumors; however, it did not bind to normal colonic tissues in humans.¹⁷ Our latest studies using an in vivo imaging apparatus and a fluorescence endoscope demonstrated that the nanobeacon illuminated colorectal cancer on the mucosa in real time.^{18,19}

The potential of the nanobeacon as an imaging tool that plays a role in the early detection of colorectal cancer and prediction of its progression has been thoroughly validated in numerous cell and animal studies.^{11–19} We are confident that the nanobeacon can be used clinically as a colonoscopic imaging agent. However, several issues must be addressed for the initiation of clinical trials. One of the most important issues is the safety of the nanobeacon. Our previous studies revealed that the nanobeacon remained locally on the luminal side of the large intestine without any systemic exposure and local irritation.^{14,17} The scientific findings indicated that the nanobeacon was a safe compound. However, regulatory agencies exercise considerable cautions before approving the human use of novel nano-sized biomaterials due to their potential toxicity.^{26–28} The present study was performed in facilities of the third party in accordance with Good Laboratory Practice (GLP) regulations with the aim of investigating toxicities of the nanobeacon after its repeated oral and single intrarectal administrations in rats.

Methods

Materials

N-Vinylacetamide (NVA) monomers were obtained from Showa Denko Co. (Tokyo, Japan). *t*-Butyl methacrylate (BMA) monomers and styrene were purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). Coumarin 6 and PNA were obtained from Tokyo Chemical Industries Co., Ltd. (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO), respectively. All other chemicals were commercial products of reagent grade. Styrene was purified by distillation, but other chemicals were used without further purification.

Caco-2 and Chinese Hamster Ovary (CHO) cells were furnished by RIKEN cell bank (Saitama, Japan) and DS Pharma Biomedical Co., Ltd. (Osaka, Japan), respectively. Dulbecco's Phosphate Buffered Saline (PBS with CaCl₂ and MgCl₂) was obtained from Sigma-Aldrich. Dulbecco's Phosphate Buffered Saline, Modified (PBS without CaCl₂ and MgCl₂) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from GIBCO Life Technologies (Carlsbad, CA). Ham's F-12 was obtained from DS Pharma Biomedical Co., Ltd. Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA). Nonessential amino acids (10 mM) and trypsin-EDTA (0.25% trypsin-1 mM EDTA for Caco-2 cells, 0.05% trypsin-0.53 mM EDTA for CHO cells) were obtained from GIBCO Life Technologies. Penicillin-streptomycin-amphotericin B suspension (penicillin: 10,000 U/mL, streptomycin: 10 mg/mL, amphotericin B: 25 µg/mL) and L-glutamine (200 mM) were purchased from Wako Pure Chemical Industries Co., Ltd. and DS Pharma Biomedical Co., Ltd., respectively.

Preparation of the nanobeacon

Preparation procedures of the nanobeacon have been described in our previous reports.^{11–19} The procedures consist of 4 steps: synthesis of vinylbenzyl group-terminated macromolecules, preparation of nanospheres, encapsulation of fluorescent dyes into the nanosphere core, and immobilization of lectins on the nanosphere surface. Under the Good Manufacturing Practice (GMP)-guided protocol, synthetic processes of the nanobeacon were significantly modified except for the first step. The nanobeacon used in this study was

prepared according to the latest synthetic manner. The details were described in Supplementary materials.

Characterization of the nanobeacon

Molecular weight—Mw/Mn of PNVA and PBMA (a precursor of PMAA) were determined by gel permeation chromatography. Agilent 1200 Infinity LC System (Agilent Technologies, Santa Clara, CA) and HLC-8320GPC (Tosoh, Tokyo, Japan) were used as the analytical system for PNVA and PBMA, respectively. TSKgel SuperAWM-H and TSKgel SuperMultiporeHZ-M (Tosoh) were used as the column for PNVA and PBMA, respectively. Column temperature was set to 40 °C. DMF containing lithium bromide (0.05 M) and tetrahydrofuran were used as the mobile phase for PNVA and PBMA, respectively. An injection volume and flow rate were set to 20 µL and 0.6 mL/min, respectively, for PNVA. Both parameters were 15 µL and 0.35 mL/min for PBMA. Refractive index and absorbance at 254 nm were monitored. Polystyrene with known molecular weights were used to provide calibration curves. Mw and Mn of PMAA were calculated from.

Particle size and zeta potential—The particle size was measured in divalent ions-free PBS by dynamic light scattering spectrophotometry (ELS-Z2, Otsuka Electronics Co., Osaka, Japan). Measurement time and temperature were set to 180 sec (the measurement was repeated 70 times) and 25 °C, respectively. The zeta potential was measured in divalent ions-free PBS by electrophoretic light scattering spectrophotometry (ELS-Z2) at 25 °C.

Fluorescence intensity—The nanobeacon was dispersed in purified water at a concentration of 1 mg/mL. The dispersion (0.2 mL) was put into a 96-well plate and the fluorescence intensity was measured using a microplate reader (EnSpire 2300 Multilabel Reader, Perkin Elmer Inc., Waltham, MA). Excitation and emission wavelengths were set to 494 nm and 518 nm, respectively.

Immobilized amount of PNA—The amount of PNA immobilized on the nanosphere surface was measured by a conventional ninhydrin method, as described in our previous article.¹¹

Fluorescence intensity of cells incubated with the nanobeacon—Caco-2 and CHO cells were seeded at a density of 1.0×10^5 and 6.0×10^4 cells/mL, respectively, in a flask with a surface area of 75 cm² (Corning Japan K. K., Tokyo, Japan). DMEM supplemented with 10% FBS, 0.5% penicillin-streptomycin-amphotericin B suspension, and 0.1 mM non-essential amino acids was used as the culture medium for Caco-2 cells, while Ham's F-12 supplemented with 10 mM L-glutamine was used for CHO cells. The cells were grown as a monolayer and maintained at 37 °C in a humidified atmosphere of 95% air/5% CO₂. The cells were then routinely passaged when they became 100% confluent, and cell lines with 5–25 passage numbers were used.

The culture medium was removed and cells were rinsed with divalent ions-free PBS. After the treatment of cells with 5 mL of trypsin-EDTA at 37 °C for 5 min, the solution was removed and the harvested cells were suspended in the culture medium (5 mL). The

suspension was centrifuged at 180 g for 5 min and the precipitated cells were re-suspended in PBS with CaCl₂ and MgCl₂ at a concentration of 1.0×10^6 cells/mL.

The nanobeacon was dispersed in divalent ions-containing PBS at a concentration of 20 mg/mL. Either Caco-2 or CHO cells (100 μ L) was mixed gently with the nanobeacon dispersion (the solution form: suspension, 33 μ L). After the mixture was incubated on ice for 1 h, it was centrifuged at 180 g for 3 min at 4 °C to separate the cells from unbound nanobeacon, and the nanobeacon-bound cells were re-suspended in ice-cooled divalent ions-containing PBS (0.45 mL). The above-mentioned cell washing process was repeated 2 times. The collected cells were finally re-suspended in ice-cooled divalent ions-containing PBS (0.35 mL). The fluorescence intensity of cell suspensions was measured using a fluorescence-activated cell sorter (Guava easyCyte 6HT-2L, Merck KGaA, Darmstadt, Germany). Excitation and emission wavelengths were set to 488 nm and 525 nm, respectively. After the mean fluorescence intensity was quantitatively estimated using the image analysis tool (Guava soft 2.3.3, Guava Express Pro, Millipore Co., Hayward, CA), it was corrected on the basis of cell size. The ratio of the corrected mean fluorescence intensity of Caco-2 cells to that of CHO cells was calculated.

Animal studies

Maintenance—Animal studies were approved by the Institutional Ethical Review Committee (approval no.: IACUC362-001) and performed in accordance with the animal welfare bylaws of Drug Safety Research Laboratories of Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan). Rats (CrI: CD (SD), 5 weeks, 108–119 g, male, n=18, Charles River Laboratories Japan, Inc., Shiga, Japan) were quarantined and acclimated in a room of GLP facilities of Shin Nippon Biomedical Laboratories, Ltd. with free access to food (laboratory animal diet, CRF-1, Lot No.: 130904 and 131008, Oriental Yeast Co., Ltd., Tokyo, Japan) and water for 7 days after they were transferred to the facility. Water, which was content to the quality standards required by the Japanese Waterworks Law, was used. Stratified randomization of the body weight (MiTOX System, Version 2.0, Mitsui Zosen System Research Inc., Chiba, Japan) was applied to select 12 rats for the toxicity study on oral administration of the nanobeacon. The residual 6 rats were subsequently maintained for 4 days and used for the toxicity study on intrarectal administration. During the study period, temperature and humidity in the room remained constantly in the range of 22.1 °C to 22.8 °C and 45% to 51%, respectively. The room was lighted and darkened at 12-h intervals and the ventilation was set to 15 times/h. The room was cleaned and disinfected every day. Each rat was kept in a stainless steel animal cage (300 mm depth $\times$ 170 mm width $\times$ 180 mm height) with bedding (chopped floor materials made of aspen, Palmas μ , Material Research Center Co., Ltd., Tokyo, Japan). Both cage and bedding were replaced with new ones autoclaved (121 °C, 30 min) when rats were allocated to the respective toxicity studies.

Oral administration of the nanobeacon—Twelve rats were divided into 2 groups (n=6, each). The nanobeacon, which was dispersed in divalent ions-containing PBS at a concentration of 50 mg/mL, was orally administered to 6 rats (the nanobeacon group) once a day for 7 days (days 0, 1, 2, 3, 4, 5, and 6) without anesthesia using a gastric catheter for rats

and a syringe at a volume of 20 mL of the dispersion per kg of body weight. Six rats assigned to the control group was treated with nanobeacon-free PBS in the same manner as described above. A day after the final administration (day 7), rats were anesthetized through intraperitoneal injection of sodium pentobarbital (6.48 mg/mL, 5 mL/kg), and approximately 2.5 mL and 2.0 mL of blood were collected from the abdominal vena cava and abdominal aorta, respectively. After rats were sacrificed by exsanguination, the esophagus, stomach, small intestine, large intestine (from cecum to rectum), liver, kidneys, and mesenteric lymph nodes were removed. All organs and tissues were fixed with 10% formaldehyde aqueous solution.

Intrarectal administration of the nanobeacon—Rats were fasted for approximately 18 h before intrarectal administration of the nanobeacon. After removal of stools from the rectum, rats were divided into 2 groups (n=3, each). Under isoflurane anesthesia (2–4%), the nanobeacon dispersion (50 mg/mL, dispersed in divalent ions-containing PBS) was intrarectally administered to 3 rats (the nanobeacon group) in a single administration (day 0) using a gastric catheter for mice and a syringe at a volume of 1 mL of the dispersion per kg of body weight. Three rats assigned to the control group was treated with nanobeacon-free PBS in the same manner as described above. All rats were fasted for 2 h after intrarectal administration. Next day (day 1), rats were sacrificed, and samples (blood, organs, etc.) were prepared as described in the previous section.

Evaluation of the toxicity of the nanobeacon

Clinical signs—All rats were physically checked once a day during the maintenance period and 3 times a day during the dosage period (before administration, approximately 1 and 4 h after administration of the nanobeacon). The final check was performed on the day of sacrifice (the group of oral administration: day 7, the group of intrarectal administration: day 1).

Body weight—Weights of all rats were recorded on the first day and the last day of the maintenance (day –1: one day before administration of the nanobeacon). Weights of rats used for the toxicity study on oral administration were subsequently monitored on days 0, 3, and 6. Weights of rats used for the toxicity study on intrarectal administration were monitored on day 0. The final weight monitor was performed on the day of sacrifice.

Food and water consumption—Food consumption of rats used for the toxicity study on oral administration was monitored on days –2 (two days before administration of the nanobeacon), 0, 3, and 5. The difference between the food amount given to each rat and the residual amount on the next day was regarded as the food consumption. A similar monitor on water consumption of the rats was performed.

Hematology—Blood samples collected from the abdominal vena cava (approximately 1 mL) were treated with EDTA potassium salt. Hematologic parameters in the whole blood, such as erythrocyte count, hematocrit value, etc. (Table 2), were measured using a hematology analyzer (ADVIA 120, Siemens Healthcare Diagnostics Manufacturing Ltd., Surrey, United Kingdom). Separately, blood samples collected from the abdominal vena

cava (approximately 1.5 mL) were mixed with 0.15 mL of sodium citrate (3.8%, w/v) and centrifuged under room temperature (1710 g, 10 min). Plasma obtained was used to determine prothrombin time and activated partial thromboplastin time using an automated blood coagulation analyzer (CA-7000, Sysmex Co., Hyogo, Japan).

Blood biochemistry—Blood samples collected from the abdominal aorta was left at room temperature for 20–60 min, and then centrifuged as described in the previous section. The resulting sera were provided to measure blood biochemical parameters, such as aspartate transaminase, alanine transaminase, etc. (Table 3), using a clinical chemistry analyzer (JCA-BM6070, JEOL Ltd., Tokyo, Japan). Protein fractions were measured using an automated electrophoresis system (AES320, Beckman Coulter Biomedical K. K., Tokyo, Japan).

Necropsy—All external and internal organs and tissues of rats sacrificed were visually examined.

Histopathology—Formaldehyde-fixed organs and tissues were embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE). Each of specimens was evaluated histopathologically. Histopathologic evaluation of the colon and the rectum was performed for rats to which the dosage solution was given intrarectally.

Statistics—Unpaired Student's *t* test was performed to calculate *p* values, and the values of 0.05 or less were considered to be statistically significant.

Results

Characteristics of the nanobeacon

Table 1 shows characteristics of the nanobeacon used in this study. The preparation and characterization of the nanobeacon were not controlled by GLP regulations; however, the nanobeacon was characterized before and after animal studies, as specified in the regulations. The characteristics of the nanobeacon were identical to those described in our previous reports.^{11–19} No significant difference was observed in any of these parameters during animal studies.

Prior to animal studies (2 weeks before commencement of the study on oral administration), the nanobeacon was initially characterized, except for the fluorescence intensity of cells incubated with the nanobeacon (ratio of mean fluorescence intensity in Table 1), which is a parameter that indicates recognition of the nanobeacon for Gal- β (1–3)GalNAc. The nanobeacon dispersion was stored at 4 °C during animal studies and the residual dispersion was retained similarly until post-characterization of the nanobeacon was performed (about 1.5 month after rat sacrifice, 2 months after initial characterization). The same batch of the nanobeacon administered to rats was stored in a lyophilized form at –20 °C as stock before animal studies were initiated. In order to substitute for the exception mentioned above, the lyophilized stock was re-dispersed and the fluorescence intensity of cells incubated with the nanobeacon was measured on the day of post-characterization. Furthermore, the stability of the nanobeacon dispersion over the course of 7 months stored in a refrigerator was validated

(Table 1, a batch used for stability studies). Properties of the nanobeacon measured were within their tentative specifications.

Toxicity of the nanobeacon

Oral administration of the nanobeacon—Any abnormality in clinical signs was not observed during the dosage period, irrespective of the dosage solution. As shown in Fig. 1A, the change of body weights of rats to which the nanobeacon had been administered was identical to that of rats to which nanobeacon-free PBS had been administered. No significant difference in food consumption was noted between each sampling point for the nanobeacon and control groups (Fig. 2A). A similar result was obtained for water consumption (Fig. 2B). No significant difference in hematologic and blood biochemical parameters was observed between the nanobeacon and control groups (Tables 2 and 3). Necropsy revealed that there was no macroscopic nanobeacon-induced change in any organs of rats. The lack of a right-side kidney observed in one rat assigned to the nanobeacon group was regarded as a congenital defect. Histopathological examination demonstrated that the nanobeacon did not induce any toxicity to digestive organs through its local exposure. Similarly, no nanobeacon-related toxicity was noted in the liver, kidneys, and mesenteric lymph nodes, which would be systemically exposed to the nanobeacon if the probe was absorbed from the intestine. Microgranuloma observed in the liver of one rat assigned to the nanobeacon group was not considered to be treatment-related because this finding is sometimes seen in normal rats.

Intrarectal administration of the nanobeacon—No abnormality in clinical signs was observed in any of the rats on the day of administration. A similar result was obtained the next day, except that the nanobeacon-like yellow-green color was slightly observed in the stools of one rat to which the nanobeacon had been administered. No nanobeacon-induced body weight change was noted (Fig. 1B). When hematologic and blood biochemical parameters of rats assigned to the nanobeacon group were compared with those assigned to the control group, 4 parameters including erythrocyte count, lymphocyte ratio, activated partial thromboplastin time, and creatinine, significantly elevated after intrarectal administration of the nanobeacon (Tables 2 and 3). However, as shown in Table 4, levels of the parameters observed in individual rats were within the standard range calculated from the clinical data for about 300 normal rats (mean $\pm$ 2s.d.). Necropsy and histopathological examination revealed that there was no macroscopic and microscopic abnormalities in any organs examined.

Discussion

The nanobeacon is a candidate of diagnostic agents for colorectal cancer combined with colonoscopy, which has been used as a definitive diagnostic tool for the cancer.^{29–31} Magnifying endoscopy and novel imaging strategies for endoscopy have enabled physicians to detect small changes on the mucosal surface of the large intestine.³² However, endoscopic screening is still based on morphological changes visualized through a macroscopic view of the mucosal surface, regardless of advance of the equipment. Tissues that look abnormal are sampled under endoscopic observation and histological evaluation is successively performed to diagnose the presence or absence of the cancer.¹⁸ There is an obvious need for the

development of a colonoscopic imaging agent that allows physicians to do real-time diagnosis of early-stage primary colorectal cancer at the molecular level. Such an imaging agent can become a useful diagnostic tool for minute tumors that are currently undetectable or difficult to detect with current endoscopic techniques. However, those agents have not been developed yet.³³

The nanobeacon used in this study was prepared in accordance with the GMP protocol. The GMP-guided modifications of synthetic procedures were successfully performed without significant influences on physicochemical and biological properties of the nanobeacon. All characteristics of the nanobeacon were also constant during the animal studies (Table 1). There are 3 differences between the previous and the latest preparation methods of the nanobeacon: the encapsulation process of coumarin 6, the purification process of particulate materials, and the immobilization process of PNA on the surface of fluorescent nanospheres. Previously, the dye encapsulation was concomitant with the particle formation through copolymerization between styrene and vinylbenzyl-terminated macromolecules, particulate materials were purified through repeated centrifugation and re-dispersion in fresh media, and lectins were immobilized by coupling their amino groups with the carboxyl groups of PMAA activated by pre-incubation with EDC.^{11–19} In the latest method, the encapsulation process of coumarin 6 was separated from the copolymerization process. Nanospheres were dispersed in ethanol dissolving coumarin 6, and water was added dropwise to the solution. The change of solvent components resulted in a reduction of the solubility of hydrophobic coumarin 6. Since the chemical potential of coumarin 6 based on hydrophobic interactions with polystyrene of the nanosphere core became less than that based on solubility in water-mixed ethanol, the dye consequently moved to the polystyrene core. An increase in the encapsulation efficiency of coumarin 6 was simultaneously observed through the process modification. The consecutive dialysis was utilized as the purification process for particulate materials because the approach used previously was impractical from a production perspective. This modification prevented particulate materials from aggregating during purification. A content of unreacted styrene monomers was less than 0.05%, which is a reporting threshold of organic impurities from a GMP perspective (data not shown). We succeeded in obtaining fluorescent nanospheres *N*-hydroxysuccinimide ester as a stable solid, which enabled us to immobilize PNA instantly on the nanosphere surface by mixing the activated nanospheres and lectins in an ice-cooled aqueous solution.

When the nanobeacon is clinically used, it will be sprayed on the large intestinal mucosa and maintained for a period that is required for its biorecognition of colorectal tumors. The nanobeacon that does not interact with tumors will be subsequently washed out with adequate solvent. The nanobeacon-bound tumors on the mucosa will be directly detected from the luminal side using a fluorescence endoscope.¹⁸ The nanobeacon has been designed as a nonabsorbable imaging agent capable of being administered topically. The frequency of its use in cancer diagnosis is predicted to be once or twice annually. In the present study, a couple of animal studies (oral and intrarectal administrations) were performed to estimate topical and systemic toxicities of the nanobeacon. The nanobeacon was administered orally to rats for 7 consecutive days at a dose of 1000 mg/kg/day. The administration schedule was designed on the basis of International Conference on Harmonization (ICH) recommendation

for non-clinical studies to support exploratory clinical trials.³⁴ The nanobeacon dispersion was substituted with PBS used as a dispersion medium in the control experiment, as generally done. No nanobeacon-induced change was observed nevertheless rats were exposed to a large amount of the nanobeacon (Figs. 1 and 2, Tables 2 and 3). It is impractical to validate non-absorbability of the nanobeacon because we should be always concerned about lower limit of quantification. Data strongly support that the nanobeacon is safe even if bodies are systemically exposed to the nanobeacon, relative substances, or degraded products that are expected to be rarely absorbed from the intestine. The no observable adverse effect level (NOAEL) of the nanobeacon was estimated to be more than 1000 mg/kg/day when administered orally every day for 7 days. Based on GLP regulations, we will be required to do a 1-month chronic toxicity of the nanobeacon before commencing clinical trials. However, administration intervals of the nanobeacon should be discussed with authorities, taking the frequency of its clinical use into consideration. We consider that the weekly administration is enough for evaluation of the chronic toxicity. Based on the regulations, the number of animals and sex will be also redesigned for the study (n = 10, each sex).

The nanobeacon was also administered intrarectally to rats. The dose was set to 50 mg/kg, considering the highest concentration of the nanobeacon in PBS with acceptable dispersibility (50 mg/mL) and the largest volume of solution capable of being administered intrarectally to rats used (1 mL/kg). Statistical differences were noted in part of hematologic and blood biochemical parameters between the nanobeacon and control groups when a single administration was performed, as expected to be done in clinics (Tables 2 and 3). However, the levels observed in individual rats were within the standard range (Table 4). It was obvious that these trivial changes unrelated to intrarectal administration of the nanobeacon. The NOAEL of the nanobeacon was estimated to be more than 50 mg/kg/day when administered once intrarectally.

We have developed nude rats in which the TF antigen-expressing tumors are present on the colonic mucosa.¹⁹ In vivo endoscopic studies are currently performed with the aim of estimating the imaging abilities of the nanobeacon in animal models that better reflect clinical environments. The latest data demonstrated that the nanobeacon-bound tumors could be detected under endoscopic observation when the nanobeacon was administered intracolonicly at a dose of 20 mg/10 mL/kg.¹⁹ This amount was significantly lower than the NOAEL observed in the present study. The results indicate that the nanobeacon can be used safely in clinical environments. The indication also accelerates our research. The overall safeties of the nanobeacon described in this report will be followed by various toxicity studies including cell toxicity assays, toxicokinetics, and so on. GMP-guided specifications and test methods for the nanobeacon and the relative substances (impurities) including degradation products are currently sophisticated to validate chemistry, manufacturing, and control (CMC) of the nanobeacon. The methods may be utilized to evaluate absorption, distribution, metabolism, and elimination (ADME) of the nanobeacon and its residues. Further works and their associated results will be reported in a timely manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.

Changes of body weights of rats before and after oral (A) and intrarectal (B) administration of either the nanobeacon (■) or nanobeacon-free PBS (○). The nanobeacon dispersed in PBS with CaCl_2 and MgCl_2 was orally administered to rats once a day for 7 days at a dose of 1000 mg of the nanobeacon/20 mL of the dispersion/kg of body weight. The same dispersion of the nanobeacon was intrarectally administered to rats in a single administration at a dose of 50 mg/mL/kg. Rats assigned to the control group was treated with nanobeacon-free PBS in the same manner as described above. Body weights were monitored on one day before administration, the day of the first administration, and the day of sacrifice. Body weights were also measured 2 times before sacrifice when the dosage solution was orally given to rats. Each value represents the mean $\pm$ s.d. of 6 (oral administration) and 3 (intrarectal administration) rats.

Fig. 2.

Changes of food (A) and water (B) consumptions after oral administration of either the nanobeacon (●) or nanobeacon-free PBS (○). The nanobeacon dispersed in PBS with CaCl_2 and MgCl_2 was orally administered to rats once a day for 7 days at a dose of 1000 mg of the nanobeacon/20 mL of the dispersion/kg of body weight. Rats assigned to the control group was treated with nanobeacon-free PBS in the same manner as described above. Both consumptions were monitored on 2 days before administration, the day of the first administration, and a couple of days after the first administration. Each value represents the mean $\pm$ s.d. of 6 rats.

Table 1

Characteristics of the nanobeacon used for animal and stability studies.

Characteristics	A batch used for animal studies		A batch used for stability studies	
	Initial characterization (Before studies)	2 month storage ^f (After studies)	Initial characterization	7 month storage ^f
Average molecular weight of PNVA (Mw/Mn)	16300/7200	n. t.e	16900/5100	n. t.e
Average molecular weight of PMAA (Mw/Mn)	12200/6500	n. t.e	12200/6500	n. t.e
Particle size (nm) ^a	382 ± 113	386 ± 103	367 ± 113	368 ± 135
Fluorescence Intensity ^b	(4.2 ± 0.1) × 10 ⁵	(4.0 ± 0.2) × 10 ⁵	(5.1 ± 0.1) × 10 ⁵	(5.0 ± 0.1) × 10 ⁵
Immobilized PNA (µg/mg) ^c	11.2	11.7	5.0	4.9
Ratio of mean fluorescence intensity (MFI) ^d	2.0 (2783/1370)	1.7 (1807/1080)	1.5 (1490/1001)	1.7 (1390/821)

^a Weight-average diameter (mean ± s.d., n = 70).

^b Fluorescence intensity of the nanobeacon dispersion (1 mg/mL, 0.2 mL) (mean ± s.d., n = 9).

^c Immobilized amount (µg) of PNA per milligram of the nanobeacon (n = 1).

^d MFI of Caco-2 cells incubated with the nanobeacon/MFI of CHO cells incubated with the nanobeacon (mean, n = 2).

^e Not tested

^f The nanobeacon dispersion was stored in a refrigerator for 2 and 7 months after initial characterization.

Table 2

Hematologic data in rats to which either PBS or the nanobeacon was administered.

Parameters	Standard				Oral administration (mean $\pm$ s.d., n = 6)				Intrarectal administration (mean $\pm$ s.d., n = 3)			
	unit	no.a	mean $\pm$ s.d.	mean - 2s.d.	mean + 2s.d.	min.b	max.c	PBS	Nanobeacon	p value	PBS	Nanobeacon
Erythrocyte count	106/ μ L	334	7.128 $\pm$ 0.404	6.320	7.936	6.18	8.91	6.905 $\pm$ 0.162	6.792 $\pm$ 0.384	0.522	6.700 $\pm$ 0.180	7.170 $\pm$ 0.217
Hemoglobin conc.	g/dL	334	14.95 $\pm$ 0.77	13.41	16.49	13.0	18.0	14.97 $\pm$ 0.37	14.60 $\pm$ 0.48	0.166	14.80 $\pm$ 0.26	15.27 $\pm$ 0.68
Hematocrit value	%	334	43.98 $\pm$ 2.38	39.22	48.74	38.1	54.0	44.83 $\pm$ 1.32	44.12 $\pm$ 1.71	0.439	45.40 $\pm$ 0.70	46.63 $\pm$ 1.93
Mean corpuscular volume	fL	334	61.74 $\pm$ 2.06	57.62	65.86	56.3	68.8	64.93 $\pm$ 1.12	65.02 $\pm$ 2.37	0.935	67.83 $\pm$ 2.84	65.00 $\pm$ 0.82
Mean corpuscular hemoglobin	pg	334	20.99 $\pm$ 0.81	19.37	22.61	18.8	24.0	21.68 $\pm$ 0.47	21.53 $\pm$ 0.82	0.706	22.13 $\pm$ 0.95	21.27 $\pm$ 0.35
Mean corpuscular hemoglobin conc.	g/dL	334	34.00 $\pm$ 1.05	31.90	36.10	31.6	36.3	33.40 $\pm$ 0.38	33.10 $\pm$ 0.32	0.170	32.63 $\pm$ 0.21	32.77 $\pm$ 0.38
Reticulocyte ratio	%	334	4.14 $\pm$ 0.85	2.44	5.84	2.2	6.9	6.32 $\pm$ 0.46	6.82 $\pm$ 0.43	0.080	5.97 $\pm$ 0.81	5.40 $\pm$ 1.01
Platelet count	103/ μ L	334	1300.5 $\pm$ 167.3	965.9	1635.1	792	1779	1352.0 $\pm$ 69.8	1324.5 $\pm$ 121.9	0.642	1460.3 $\pm$ 25.3	1394.3 $\pm$ 166.2
Leukocyte count	103/ μ L	334	7.805 $\pm$ 1.954	3.897	11.713	3.80	14.31	9.778 $\pm$ 1.434	9.603 $\pm$ 2.554	0.887	6.300 $\pm$ 0.436	8.340 $\pm$ 1.394
Neutrophil count	103/ μ L	334	1.076 $\pm$ 0.409	0.258	1.894	0.35	2.88	1.182 $\pm$ 0.437	1.053 $\pm$ 0.267	0.551	0.460 $\pm$ 0.061	0.460 $\pm$ 0.072
Lymphocyte count	103/ μ L	334	6.442 $\pm$ 1.729	2.984	9.900	2.97	12.48	8.175 $\pm$ 1.108	8.162 $\pm$ 2.281	0.990	5.640 $\pm$ 0.457	7.647 $\pm$ 1.305
Monocyte count	103/ μ L	334	0.131 $\pm$ 0.063	0.005	0.257	0.04	0.50	0.200 $\pm$ 0.094	0.178 $\pm$ 0.041	0.611	0.057 $\pm$ 0.023	0.083 $\pm$ 0.032
Eosinophil count	103/ μ L	334	0.063 $\pm$ 0.038	< 0	0.139	0.01	0.55	0.053 $\pm$ 0.014	0.057 $\pm$ 0.015	0.643	0.047 $\pm$ 0.006	0.057 $\pm$ 0.021
Basophil count	103/ μ L	334	0.025 $\pm$ 0.014	< 0	0.053	0.00	0.09	0.032 $\pm$ 0.012	0.030 $\pm$ 0.015	0.804	0.017 $\pm$ 0.012	0.017 $\pm$ 0.006
Large unstained cell count	103/ μ L	334	0.067 $\pm$ 0.037	< 0	0.141	0.01	0.29	0.133 $\pm$ 0.052	0.122 $\pm$ 0.039	0.687	0.083 $\pm$ 0.025	0.083 $\pm$ 0.021
Neutrophil ratio	%	334	14.02 $\pm$ 4.74	4.54	23.50	5.0	32.3	12.00 $\pm$ 3.72	11.17 $\pm$ 2.39	0.655	7.37 $\pm$ 1.45	5.50 $\pm$ 0.36
Lymphocyte ratio	%	334	82.33 $\pm$ 4.98	72.37	92.29	63.8	92.7	83.80 $\pm$ 4.36	84.80 $\pm$ 2.61	0.640	89.47 $\pm$ 1.05	91.63 $\pm$ 0.40
Monocyte ratio	%	334	1.67 $\pm$ 0.58	0.51	2.83	0.5	4.1	1.98 $\pm$ 0.67	1.88 $\pm$ 0.32	0.748	0.97 $\pm$ 0.32	1.00 $\pm$ 0.30
Eosinophil ratio	%	334	0.82 $\pm$ 0.41	< 0	1.64	0.2	6.0	0.55 $\pm$ 0.16	0.57 $\pm$ 0.10	0.800	0.73 $\pm$ 0.06	0.63 $\pm$ 0.21
Basophil ratio	%	334	0.31 $\pm$ 0.15	0.01	0.61	0.0	1.1	0.33 $\pm$ 0.05	0.30 $\pm$ 0.06	0.369	0.27 $\pm$ 0.12	0.20 $\pm$ 0.10
Large unstained cell ratio	%	334	0.84 $\pm$ 0.42	< 0	1.68	0.2	5.5	1.32 $\pm$ 0.32	1.28 $\pm$ 0.23	0.809	1.27 $\pm$ 0.35	1.00 $\pm$ 0.36
Prothrombin time	s	206	15.81 $\pm$ 3.37	9.07	22.55	9.6	30.4	15.12 $\pm$ 3.16	12.55 $\pm$ 1.46	0.101	12.50 $\pm$ 0.82	13.77 $\pm$ 2.08
Activated partial thromboplastin time	s	297	22.17 $\pm$ 3.41	15.35	28.99	14.9	40.1	23.50 $\pm$ 4.88	20.23 $\pm$ 3.43	0.209	19.87 $\pm$ 1.67	24.53 $\pm$ 1.63

^aThe number of rats used for calculation of the standard (Cri: CD (SD), 7–8 weeks, male).

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The minimal value of each parameter observed in rats used for calculation of the standard.
The maximal value of each parameter observed in rats used for calculation of the standard.
* Statistically significant difference between control (PBS) and the nanobeacon groups

Table 3

Blood biochemical data in rats to which either PBS or the nanobeacon was administered.

Parameters	unit	no.a	Mean ± s.d.	mean - 2×s.d.	mean + 2×s.d.	min.b	max.c	Oral administration (mean ± s.d., n = 6)				Intrarectal administration (mean ± s.d., n = 3)			
								PBS	Nanobeacon	P	P value	PBS	Nanobeacon	P	P value
Aspartate transaminase	IU/L	375	88.8 ± 18.8	51.2	126.4	55	169	77.7 ± 22.7	72.7 ± 8.3	0.630	0.630	98.0 ± 22.7	75.7 ± 8.3	0.185	0.185
Alanine transaminase	IU/L	385	28.1 ± 4.7	18.7	37.5	18	48	34.5 ± 3.9	35.8 ± 4.3	0.595	0.595	37.0 ± 2.6	30.0 ± 5.3	0.109	0.109
Alkaline phosphatase	IU/L	375	800.2 ± 175.3	449.6	1150.8	464	1736	891.8 ± 233.1	930.7 ± 224.4	0.774	0.774	846.3 ± 169.9	875.0 ± 145.7	0.835	0.835
Creatine kinase	IU/L	312	455.1 ± 216.5	22.1	888.1	158	1235	435.8 ± 283.0	372.8 ± 89.8	0.622	0.622	790.3 ± 327.5	509.0 ± 131.9	0.240	0.240
Total bilirubin	mg/dL	375	0.034 ± 0.012	0.010	0.058	0.00	0.07	0.035 ± 0.010	0.038 ± 0.008	0.579	0.579	0.013 ± 0.015	0.023 ± 0.021	0.539	0.539
Total protein	g/dL	355	5.29 ± 0.24	4.81	5.77	4.7	6.3	5.37 ± 0.14	5.32 ± 0.10	0.493	0.493	4.93 ± 0.21	4.97 ± 0.15	0.802	0.802
Triglyceride	mg/dL	355	30.1 ± 12.9	4.3	55.9	9	113	36.0 ± 11.0	33.2 ± 7.0	0.610	0.610	21.0 ± 7.5	26.3 ± 16.2	0.634	0.634
Total cholesterol	mg/dL	355	53.2 ± 11.4	30.4	76.0	22	95	73.7 ± 11.8	79.2 ± 15.3	0.502	0.502	70.3 ± 9.5	68.0 ± 19.5	0.863	0.863
Glucose	mg/dL	355	131.2 ± 14.6	102.0	160.4	87	190	137.3 ± 15.5	139.8 ± 7.3	0.728	0.728	130.7 ± 10.7	142.3 ± 15.0	0.337	0.337
Urea nitrogen	mg/dL	375	15.16 ± 2.66	9.84	20.48	9.7	31.0	22.20 ± 3.76	22.03 ± 2.76	0.931	0.931	14.23 ± 0.95	19.03 ± 5.15	0.188	0.188
Creatinine	mg/dL	375	0.193 ± 0.029	0.135	0.251	0.12	0.29	0.215 ± 0.014	0.213 ± 0.015	0.816	0.816	0.187 ± 0.015	0.213 ± 0.006	0.049*	0.049*
Inorganic phosphorus	mg/dL	355	9.181 ± 0.779	7.623	10.739	7.60	12.54	10.307 ± 0.640	10.205 ± 0.609	0.783	0.783	10.367 ± 0.313	10.403 ± 0.147	0.866	0.866
Ca	mg/dL	355	9.68 ± 0.28	9.12	10.24	8.6	10.5	10.15 ± 0.23	10.23 ± 0.18	0.517	0.517	9.63 ± 0.12	9.90 ± 0.46	0.381	0.381
Na	mEq/L	355	143.6 ± 1.7	140.2	147.0	139	148	141.2 ± 1.2	141.0 ± 0.6	0.723	0.723	142.0 ± 1.0	141.0 ± 1.0	0.288	0.288
K	mEq/L	355	4.01 ± 0.32	3.37	4.65	3.2	6.6	4.25 ± 0.30	4.23 ± 0.21	0.896	0.896	4.20 ± 0.36	4.43 ± 0.31	0.449	0.449
Cl	mEq/L	355	105.6 ± 2.2	101.2	110.0	100	113	103.3 ± 1.4	102.5 ± 1.4	0.346	0.346	105.3 ± 0.6	105.7 ± 0.6	0.460	0.460
Albumin ratio	%	339	55.73 ± 2.27	51.19	60.27	49.7	66.7	58.93 ± 1.77	58.83 ± 1.79	0.924	0.924	59.33 ± 1.50	60.07 ± 2.47	0.680	0.680
γ-1 globulin ratio	%	339	19.33 ± 2.19	14.95	23.71	11.9	25.2	18.47 ± 1.58	18.17 ± 0.96	0.699	0.699	18.27 ± 0.49	16.90 ± 0.92	0.085	0.085
γ-2 globulin ratio	%	339	7.37 ± 0.87	5.63	9.11	4.5	9.7	5.98 ± 0.59	6.28 ± 0.87	0.500	0.500	6.83 ± 0.49	7.07 ± 0.78	0.675	0.675
γ-globulin ratio	%	339	13.91 ± 0.82	12.27	15.55	11.0	15.9	14.00 ± 0.54	14.02 ± 0.95	0.965	0.965	12.20 ± 1.11	12.37 ± 0.55	0.824	0.824
γ-globulin ratio	%	339	3.66 ± 0.71	2.24	5.08	2.3	6.6	2.62 ± 0.46	2.70 ± 0.23	0.711	0.711	3.37 ± 0.55	3.60 ± 0.62	0.656	0.656
A/G (Albumin/Globulin)	-	339	1.265 ± 0.123	1.019	1.511	0.99	2.00	1.440 ± 0.101	1.433 ± 0.110	0.911	0.911	1.460 ± 0.090	1.510 ± 0.157	0.657	0.657
Albumin	g/dL	339	2.95 ± 0.15	2.65	3.25	2.6	3.5	3.15 ± 0.10	3.13 ± 0.08	0.710	0.710	2.93 ± 0.06	3.00 ± 0.17	0.538	0.538
Globulin	g/dL	339	2.35 ± 0.19	1.97	2.73	1.7	3.0	2.22 ± 0.12	2.18 ± 0.13	0.592	0.592	2.00 ± 0.17	1.97 ± 0.12	0.815	0.815

^aThe number of rats used for calculation of the standard (Ctrl: CD (SD), 7–8 weeks, male)

* Statistically significant difference between control (PBS) and the nanobeacon groups

_c The maximal value of each parameter observed in rats used for calculation of the standard

_q The minimal value of each parameter observed in rats used for calculation of the standard

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Table 4

Hematologic and blood biochemical data in individual rats to which either PBS or the nanobeacon was administered intrarectally.

Parameters	Standard			PBS					Nanobeacon			
	unit	no.a	mean ± s.d.	mean- 2×s.d.	mean+ 2×s.d.	min.b	max.c	rat 13	rat 15	rat 14	rat 16	rat 18
Erythrocyte count	10 ⁶ /μL	334	7.128 ± 0.404	6.320	7.936	6.18	8.91	6.85	6.50	7.28	7.31	6.92
Lymphocyte ratio	%	334	82.33 ± 4.98	72.37	92.29	63.8	92.7	90.5	88.4	91.7	92.0	91.2
Activated partial thromboplastin time	s	297	22.17 ± 3.41	15.35	28.99	14.9	40.1	21.2	20.4	26.3	24.2	23.1
Creatinine	mg/dL	375	0.193 ± 0.029	0.135	0.251	0.12	0.29	0.19	0.17	0.22	0.21	0.21

^aThe number of rats used for calculation of the standard (Ctrl: CD (SD), 7–8 weeks, male).

^bThe minimal value of each parameter observed in rats used for calculation of the standard.

^cThe maximal value of each parameter observed in rats used for calculation of the standard.