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Preclinical Safety Pharmacology Study of a Novel Protein-Based Cancer Vaccine CHP-NY-ESO-1

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CHP-NY-ESO-1 is a novel therapeutic cancer vaccine consisting of a recombinant protein of cancer antigen NY-ESO-1 and a polysaccharide-based delivery system, cholesteryl pullulan. A pilot clinical study of CHP-NY-ESO-1 in cancer patients was previously conducted, and the adverse events related to this drug were observed to be limited to skin reactions at injection sites. To further establish the safety of CHP-NY-ESO-1, we studied the effects of its subcutaneous injection on vital functions such as the central nervous system, cardiovascular system and respiratory system using preclinical animal models. The effects of CHP-NY-ESO-1 on the cardiovascular system were investigated in dogs using a telemetry system for blood pressure and heart rate and the Holter monitoring for ambulatory electrocardiograms. No drug-related changes were observed in these parameters. The effect of CHP-NY-ESO-1 on the hERG-dependent potassium currents was also examined using *in vitro* cultured cell system, and no inhibition of hERG currents was observed. The effects of CHP-NY-ESO-1 on the central nervous system were examined in rats using functional observational battery method, and no drug-related changes were observed in home cage observations, open field observations, hand held observations, and perception and motor function observations. The effect of CHP-NY-ESO-1 on the respiratory system was investigated in rats by measuring tidal volume, minute volume and respiratory rate using whole-body plethysmograph method, and no significant changes were found in these parameters. These results indicate that CHP-NY-ESO-1 would not have any pharmacological effects on vital functions and support the safety of this cancer vaccine for clinical use.

Although there are multiple approaches to therapy and prevention, cancer remains a major cause of death worldwide, and new therapeutic approaches are needed. Since the molecular mechanisms involved in immunological recognition and eradication of tumors has been elucidated, cancer immunotherapy to utilize the power and specificity of immunity for the treatment of malignancy is emerging as an attractive new therapy (1, 2). Cancer vaccines

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to induce cancer immunity by immunizing patients with antigen(s) are representative of cancer immunotherapy. Our CHP-NY-ESO-1 is a novel cancer vaccine unique for utilizing the recombinant protein of highly immunogenic cancer antigen NY-ESO-1 as the target antigen as well as for employing a novel antigen delivery system, cholesteryl hydrophobized pullulan (CHP).

NY-ESO-1 is a tumor-associated antigen protein originally identified in human esophageal cancer (3). While the expression of NY-ESO-1 is strictly restricted to testis and ovary among adult normal tissues, it is highly expressed in a wide variety of cancers including esophagus, prostate, breast and melanoma (reviewed in ref 4). NY-ESO-1 protein is also well known to possess strong immunogenicity; spontaneous humoral response against NY-ESO-1 frequently occurs in patients with NY-ESO-1-expressing tumors, and both of $CD4^+$ and $CD8^+$ T cell responses are also found in patients serologically positive for anti-NY-ESO-1 antibody (4). Such cancer-specific expression profile and high immunogenicity of NY-ESO-1 encourage us to develop a therapeutic cancer vaccine utilizing this protein as the target antigen. On the other hand, CHP consists of a polysaccharide pullulan containing chemically introduced cholesterol groups to add some hydrophobicity to this polysaccharide backbone (5). Via hydrophobic interaction between the introduced cholesterol groups, CHP spontaneously aggregates to form nano-sized particles that can contain antigen proteins in their interiors. CHP can assist with protein solubilization and stability and also can improve *in vivo* pharmacokinetic profile of protein-based drugs (5, 6). In addition to this feature, CHP system has the ability especially applicable to the delivery of antigen proteins in cancer vaccines. It is generally understood that vaccines with protein antigens can efficiently stimulate $CD4^+$ helper T cells via the endosomal MHC class II pathway but not $CD8^+$ killer T cells via the cytosolic MHC class I pathway (7). Because it is believed that $CD8^+$ killer T cell response is crucial for anti-tumor effects (8, 9), the incapability of protein vaccines to efficiently induce specific $CD8^+$ killer T cells is a major drawback of this type of cancer vaccine. However, in a series of *in vitro* experiments and clinical researches with CHP protein vaccines, it was discovered that antigen proteins delivered by CHP into antigen-presenting cells can proficiently stimulate not only specific $CD4^+$ T cells but also specific $CD8^+$ killer T cells. An *in vitro* experiments with CHP-NY-ESO-1 showed that dendritic cells pulsed with this vaccine could induce both NY-ESO-1-specific $CD4^+$ and $CD8^+$ T cells in the blood samples from healthy donors (10). In a previous clinical study, most of the cancer patients vaccinated with CHP-NY-ESO-1 mounted NY-ESO-1-specific $CD8^+$ T cell response as well as specific $CD4^+$ T cell response (11). Similar results were also obtained with another CHP cancer vaccine, CHP-HER2, both *in vitro* and *in vivo* (12, 13). Thus, the utilization of CHP delivery system in protein vaccines can overcome the major drawback of protein vaccines, making CHP protein vaccines unique from others. In addition, in the previous pilot clinical study of CHP-NY-ESO-1, low toxicity of this vaccine and the tumor responses in some vaccinated patients were also observed (11). These findings with CHP-NY-ESO-1 vaccine obtained in the preclinical and clinical researches indicate that this vaccine could serve as a novel and effective immunotherapy for a variety of human cancers.

In order to make this cancer vaccine into the practical use, its further clinical development is planned, and to support it, a series of preclinical studies including pharmacology, toxicology, and pharmacokinetic studies has been performed. The safety pharmacological properties of CHP-NY-ESO-1 were also evaluated and the results are described in this report.

MATERIALS AND METHODS

This study was conducted in accordance with the ICH guidance for Safety Pharmacology Studies for Human Pharmaceuticals (July 2001) and in compliance with Good Laboratory Practice (GLP).

The CHP-NY-ESO-1 Vaccine. Full length NY-ESO-1 cDNA was cloned into pET vector and introduced into a strain of Escherichia coli, C41 (DE3) harboring pRARE plasmid. The expression of His_6-NY-ESO-1 protein was induced by the addition of isopropyl-L-thio-β-D-galactopyranoside to the bacterial cell culture, and the produced protein was recovered in and extracted from inclusion bodies and then highly purified using the combination of chromatographic techniques including metal chelating affinity chromatography, anion exchange chromatography and hydrophobic chromatography. CHP was synthesized by a chemical reaction between pullulan (average molecular weight of 100kDa) and cholesterol isocyanate in pyridine/dimethyl sulfoxide solution (Nippon Oil and Fat Co., Tokyo, Japan). After purification by extraction and precipitation, resultant CHP was emulsified in water and subsequently freeze-dried. When resolved into water or buffers, CHP spontaneously forms nanoparticles. These nanoparticles (20-50 nm) contain the hydrophobic domains of cholesterol groups in their inside, which associate with the hydrophobic regions of the NY-ESO-1 protein, forming a stable complex in solution. This complex of protein and CHP was used as the CHP-NY-ESO-1 vaccine [0.45 mg/mL NY-ESO-1 and 13.1 mg/mL CHP in phosphate-buffered saline (PBS)] (Figure 1).

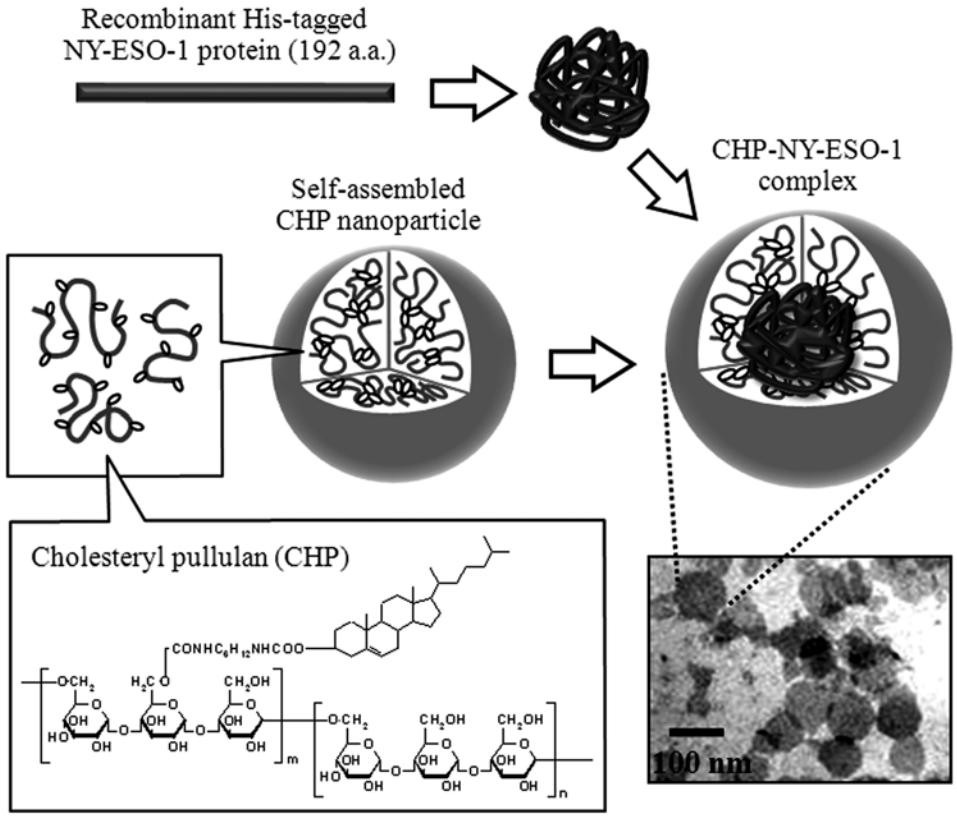

FIG. 1. Structure of CHP-NY-ESO-1 cancer vaccine. The photograph represents the transmission electron microscopic analysis of CHP-NY-ESO-1. a.a., amino acids.

Animals and Drug Administration. The experiments were conducted using male Beagle dogs (9.0 to 12.0 kg, Covance Research Product, Inc.) or male Sprague-Dawley rats (175 to 223 g, Charles River Japan, Inc.). Animals were kept in the rooms at the Animal Buildings of Kobuchisawa Research Laboratories, Fuji Biomedix Co., Ltd. under the conditions of temperature of 22 ± 4°C, relative humidity at 50 ± 20% and lighting at 12 hr light/dark cycle. Pelleted food and tap water were freely available to each animal. In all experiments except hERG assay, animals received the single subcutaneous injection of CHP-NY-ESO-1 at the doses of 0.1, 0.3 or 0.5 mg NY-ESO-1 protein/animal or PBS as a vehicle control. In this study, 0.5 mg protein/animal was employed as the high dose, based on the intended maximum clinical dose of 0.5 mg protein/body in a single dose. Then 0.3 and 0.1 mg protein/animal were selected as the middle and low doses, calculated by deducting with a number of 0.2. These lower doses were also in a range of intended clinical doses. The experiments were applied to and approved by the Animal Experiment Ethics Committee of Fuji Biomedix Co., Ltd.

Effects on Cardiovascular System in Conscious Dogs. A telemetry transmitter (TA11PA-D70, Data Sciences International, Inc.) was surgically implanted in each dog. For implantation surgery, dog was anesthetized by an intravenous injection of sodium pentobarbital solution at 30 mg/kg, and a transmitter implanted in the lateroabdominal subcutaneous region. A catheter was then inserted into the abdominal aorta through the right femoral artery via subcutis. Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure [(SBP+2DBP)/3], and heart rate were continuously measured using an automatic telemetry system (Dataquest ART platinum version 3.1, Data Sciences International Inc.) (14). For the measurement of ambulatory electrocardiograms (ECG), a jacket containing Holter's electrocardiograph (QR2100, Fukuda M-E Kogyo Co., Ltd.) was put on each dog, and ECG was recorded by M-X lead (episternum: −, xiphosternum: +) and R-L lead (right thorax: −, left thorax: +) (15). An ambulatory ECG analyzer (HS 1000 system, Fukuda M-E Kogyo Co., Ltd.) was used for the analysis. ECG parameters [QRS width, PR, QT intervals (mean value for 5 successive beats), and QTc (calculated by Fridericia's formula: $QTc=QT/\sqrt[3]{RR}$)] (16). Arrhythmia such as premature ventricular contraction was also analyzed on each sampling point of ECG. Data were collected before dosing, at 6 and 12 hours, and on 1, 2, 3, 6, 9, 12 and 15 days after dosing.

Effects on hERG-Mediated Potassium Current in HEK293 Cells. HEK293 (human embryo kidney 293) cells transfected with hERG (human ether-a-go-go-related gene) (17) were purchased from Cytomyx Ltd. and maintained in the Minimum Essential Medium containing 10% fetal bovine serum, 1% non-essential amino acids and 400 μg/mL Geneticin on collagen-coated 60 mm dishes at 37˚C under 5% CO_2 atmosphere. For the cell detachment, trypsin-EDTA (0.25% trypsin-1 mM EDTA-4Na) diluted to 0.05% with PBS (pH 7.4) was used. For the hERG current measurement, cells were cultured on collagen-coated cover glasses and set to a 35 mm dish. The perfusion was performed using a perfusate (137 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) kept at 26˚C at the flow rate of 120 mL/h. Glass electrodes were made by pulling from borosilicate glass capillaries using a horizontal micropipette puller and filled with the electrode solution (130 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 5 mM Mg-ATP, 10 mM HEPES, pH 7.2). The hERG current was then recorded by the voltage-clamp method (18) in the whole cell configuration with the EPC 8 (HEKA) under the control of pClamp 9 software (Axon Instruments). Pulses were applied at 15-second intervals by the following procedure: holding voltage at −80 mV, depolarization pulse for 1.5 sec each at 4 steps by increasing 20 mV at a time from −40 mV to +20 mV, and repolarization pulse for 1.5 sec at

−50 mV. The cells were perfused with the solution containing CHP-NY-ESO-1 (0.04, 0.15 or 0.45 μg/mL) or E-4031 (15 ng/mL, Wako Chemical) as a positive control (17). The effect of test drug was examined by measuring the peak value of tail current at +20 mV before and 10 minutes after the addition of test drug.

Effects on Central Nervous System in Conscious Rats. Rats treated with CHP-NY-ESO-1 were observed by the functional observational battery method (19, 20). Home cage observations (body posture, behavior, ease of removal from cage), open field observations (number of unit areas crossed, rearing count, number of fecal pellets, number of pools of urine, respiration rate, stereotypy, unusual behavior, tremor, convulsion, gait, arousal level), hand held observations (fur and skin appearance, piloerection, soiled lower abdominal region, lacrimation, salivation, palpebral closure, pupil size, pupillary reflex, muscle tone, extensor-thrust reflex), perception and motor function observations (visual response, touch response, click response, pain response, aerial righting reflex, hindlimb landing foot splay, grip strength) and measurement of body temperature were performed before dosing and at 6, 12, 24, 48, 72 hours and on 6, 9, 12 and 15 days after dosing.

Effects on Respiration in Conscious Rats. In the rats treated with CHP-NY-ESO-1, respiratory functions such as tidal volume, minute volume and respiratory rate were measured by the whole-body plethysmograph method (21) under non-restraint condition using the respiratory functions measurement system (Respytox-8, M.I.P.S. Co.) for 5 minutes continuously before dosing and at 6, 12, 24, 48, 72 hours and on 6, 9, 12 and 15 days after dosing. Each animal was placed in a non-restraint chamber more than 20 minutes before each time of measurement. During the measurement, clinical signs and body movement of rats were carefully observed. Animals were deprived of food and water when they were kept in a non-restraint chamber.

Statistics. Quantitative data were expressed in mean ± standard deviation. In dog cardiovascular system study, time sequential changes were analyzed by the mixed procedure to assess the effect of dose (dose response) and the interaction of dose and time after dosing (dose x time response). If significant difference was found in dose response or dose x time response, comparison between the vehicle control group and the CHP-NY-ESO-1 group was conducted by Dunnett's test. In hERG study, comparison between the vehicle control group and the positive control and between the vehicle control group and the CHP-NY-ESO-1 group was performed by Student's *t*-test and Dunnett's test, respectively. In rat studies, comparison between the vehicle control group and each dosage group of CHP-NY-ESO-1 was performed by Dunnett's test at each time point. Statistical significance was assumed when $p < 0.05$. SAS® (Windows version, Release 8.2, SAS Institute Inc.) was used for these analyses.

RESULTS

Effects on Cardiovascular System in Conscious Dogs. Measurements of blood pressure and heart rate by the telemetry method and ambulatory electrocardiography (ECG parameters and appearance of arrhythmia) using Holter monitoring were performed in the CHP-NY-ESO-1-treated dogs until 15 days after dosing. The time points of observation were decided in consideration of the course of change after dosing of the CHP-NY-ESO-1 vaccine or related CHP protein vaccine in clinical studies (11, 13). Published data on the pharmacokinetics of protein-based and liposomal drugs were also considered (22-24). The changes in blood pressure and heart rate and the changes in ECG parameters are shown in Figures 2 and 3, respectively. No effects of the CHP-NY-ESO-1 vaccine on blood pressure (systolic blood pressure, diastolic blood pressure and mean blood pressure), heart rate and

ECG parameters (PR interval, QRS width, QT interval or QTc) were found. The occurrence of arrhythmia was also analyzed in each analysis point, and the outbreak of arrhythmia was not observed (data not shown).

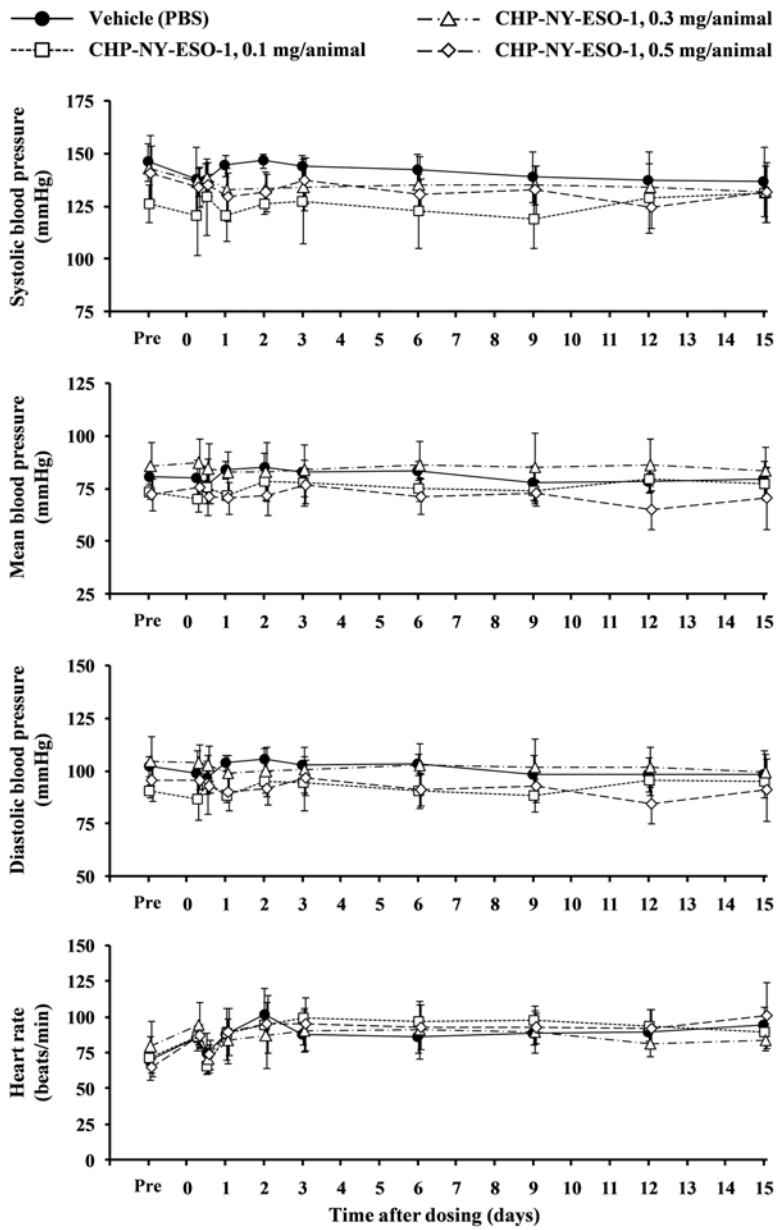

FIG. 2. The effects of subcutaneous injection of CHP-NY-ESO-1 on blood pressure and heart rate in conscious dogs. Data shown are mean ± standard deviation (n=4). No significant difference between vehicle and CHP-NY-ESO-1 (0.1, 0.3, or 0.5 mg/animal) was found (Dunnett's test, $P < 0.05$).

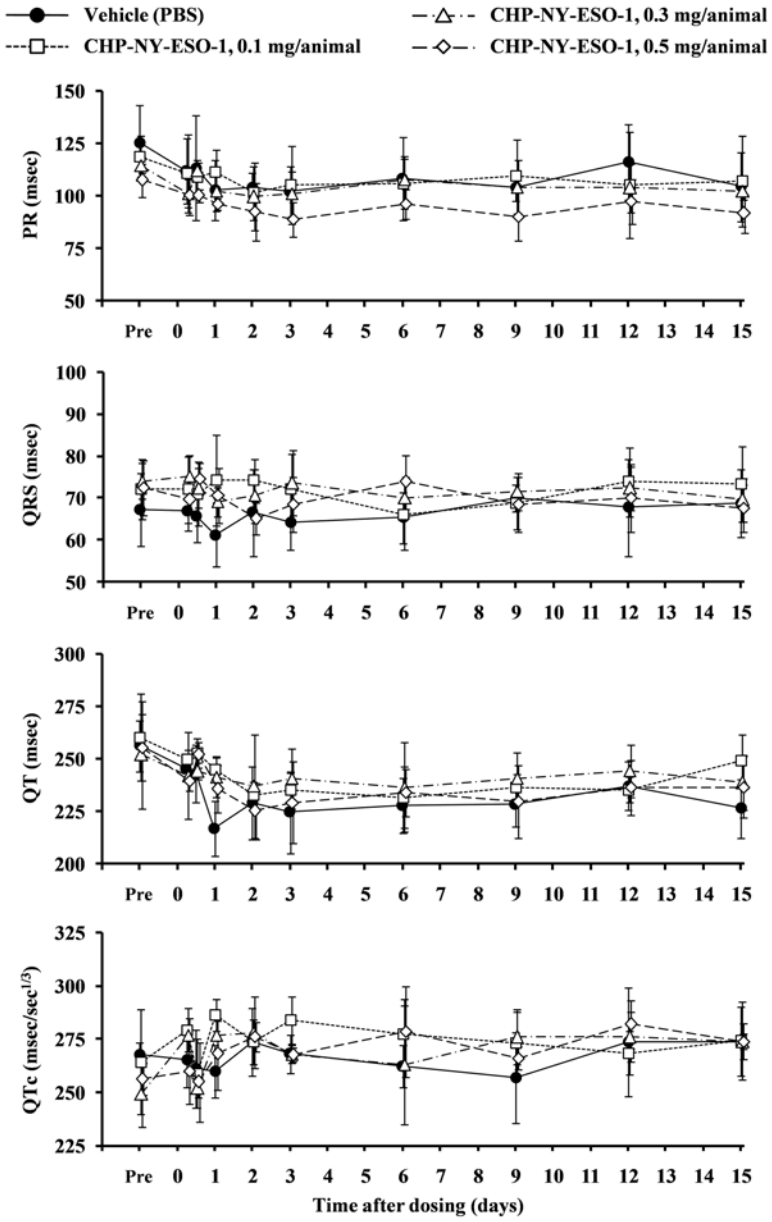

FIG. 3. The effects of subcutaneous injection of CHP-NY-ESO-1 on ECG parameters in conscious dogs. Data shown are mean ± standard deviation (n=4). No significant difference between vehicle and CHP-NY-ESO-1 (0.1, 0.3, or 0.5 mg/animal) was found (Dunnett's test, $P < 0.05$).

Effects on hERG-Mediated Potassium Current in HEK293 Cells. The data were presented as inhibition rate which means the percentage of the mean peak value of tail current after the addition of test drug relative to the value before addition of drug (Table 1). In the vehicle control group (PBS) and the positive control group (E-4031), the inhibition rate was 4.3% and 51.7%, respectively. In the CHP-NY-ESO-1 groups, the inhibition rates were 1.0%, 0.5%, and 4.1% in the groups of 0.04, 0.15 and 0.45 μg/mL, respectively. There were no statistically significant differences in the changes in any CHP-NY-ESO-1 group as compared to that in the vehicle control group.

TABLE 1. The effect of CHP-NY-ESO-1 on the potassium currents in hERG-transfected HEK293 cells.

	Concentration (μg/mL)	Inhibition (%)
Vehicle (PBS)		4.3 ± 4.0
CHP-NY-ESO-1	0.04	1.0 ± 3.8
	0.15	0.5 ± 5.9
	0.45	4.1 ± 2.2
E-4031	0.015	51.7 ± 1.1*

Data are the mean ± standard deviation of the results obtained in 5 cells. *p< 0.01, significantly different from the vehicle control group by Student's *t* test.

Effects on Central Nervous System in Conscious Rats. The effect of CHP-NY-ESO-1 on the central nervous system was evaluated in rats until 15 days after dosing using the functional observational battery method. In the groups of the doses of 0.1 and 0.3 mg/animal, no significant change was observed in any parameters after dosing. In the group of the dose of 0.5 mg/animal, the body temperature on 15 days after dosing was decreased as compared with the control group (the control group: 38.3˚C; 0.5 mg/animal of CHP-NY-ESO-1 group: 37.8˚C). However, this change was considered accidental, because the difference in body temperature between these 2 groups was as low as 0.5˚C, and the body temperature of animals in the group of the dose of 0.5 mg/animal was not different from the pre-dose value (37.8-38.1˚C). No changes were observed in other parameters.

Effects on Respiration in Conscious Rats. Using the whole-body plethysmograph method, respiratory functions (tidal volume, minute volume and respiratory rate) were investigated time-sequentially in the CHP-NY-ESO-1-treated rats until 15 days after dosing. Rats in the control group, that received PBS, were similarly evaluated. CHP-NY-ESO-1 had no significant effects on the tidal volume, minute volume, or respiratory rate in rats at any dose (Figure 4).

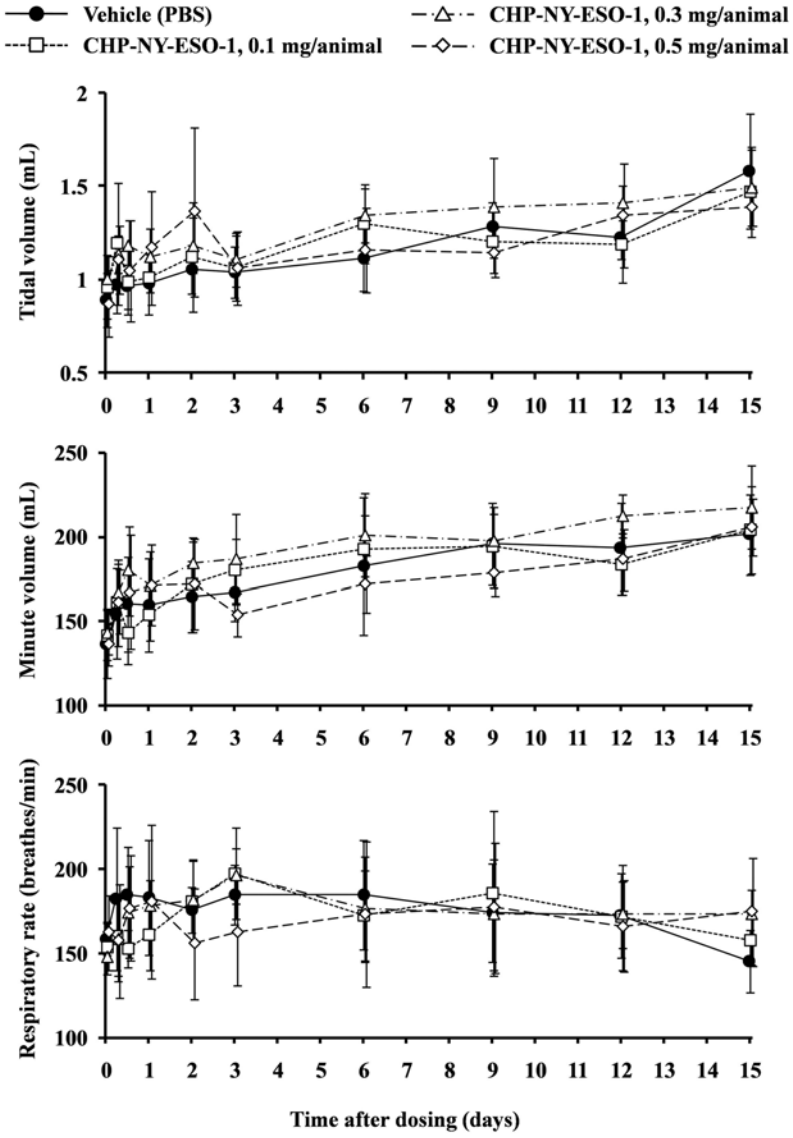

FIG. 4. The effects of subcutaneous injection of CHP-NY-ESO-1 on tidal volume, minute volume and respiration rate in conscious rats. Data shown are mean ± standard deviation (n=4). No significant difference between vehicle and CHP-NY-ESO-1 (0.1, 0.3, or 0.5 mg/animal) was found (Dunnett's test, $P < 0.05$).

DISCUSSION

In the present study, the safety pharmacological effects of the single subcutaneous injection of CHP-NY-ESO-1 vaccine on the cardiovascular system, central nervous system, and respiratory systems were investigated. The results indicated that CHP-NY-ESO-1 at any doses caused no significant changes in the functions of these systems.

Considering the route of the administration, i.e. subcutaneous injection, and the size of the CHP-NY-ESO-1 with the diameter of around 100 nm (Figure 1), it is possible that

CHP-NY-ESO-1 remains to exist at the injection site for a long period rather than easily leaks to blood stream and distribute to whole body. If it is the case, CHP-NY-ESO-1 will not reach at any organs involved in the cardiovascular system, central nervous system and respiratory system and will not affect them at all. However, recent reports on the pharmacokinetics of subcutaneously injected protein-based or liposomal drugs indicate that they can be absorbed from the injection sites, and via lymphatic capillary, then can be distributed in a variety of tissues and organs (25, 26). CHP-NY-ESO-1 also may show similar distribution profile and reach at vital organs for cardiovascular system, central nervous system, and respiratory system.

However, even if CHP-NY-ESO-1 reaches at these sites, it would be still unlikely that this vaccine directly influences the functions of these organs, because no any physiological functions are reported for both of CHP and NY-ESO-1 protein. A preclinical pharmacokinetic study of CHP-NY-ESO-1 has been conducted in parallel to this study, and its result will provide us with some clues for the explanation of the results observed in this safety pharmacology study.

On the other hand, this drug is a vaccine which is intended to elicit the antigen-specific immune responses. Induced immune responses such as cellular and humoral responses may target some of the vital organs, if these organs express target antigen. However, it is well known that NY-ESO-1 antigen is expressed only in testis and ovary in adult normal tissues, suggesting that the vital organs including heart and lung will not be targeted by the CHP-NY-ESO-1-induced immune responses.

In conclusion, the single subcutaneous administration of the CHP-NY-ESO-1 vaccine into animals caused no changes in the cardiovascular system, central nervous system, and respiratory system, supporting its safety in clinical use.

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