INTRODUCTION

The constitutive active/androstane receptor (CAR, NR1I3) is a member of the nuclear receptor family protein that functions as a xenobiotic sensor. The CAR target genes are related to xenobiotic metabolism/detoxification and excretion involving phase I oxidation enzymes (e.g., CYP family proteins), phase II conjugation enzymes (e.g., uridine 5'-diphosphate (UDP)-glucuronosyltransferases), and phase III efflux transport proteins including multidrug resistance proteins. Moreover, CAR also mediates various hepatic functions that control diverse physiological and pathophysiological processes, e.g., gluconeogenesis, fatty acid oxidation, cell apoptosis and proliferation, tumor development, insulin signaling, and the clearance of endogenous bile acids, bilirubin, and steroid hormones. Thus, the ability to either activate or repress CAR can have either protective or deleterious consequences; therefore, the production of compounds that do not bind to CAR is important in drug discovery.

CAR consists of an N-terminal DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD), which is a characteristic of other nuclear receptors including pregnane X receptor (PXR), peroxisome proliferator-activated receptor (PPAR), and estrogen receptor (ER). Upon the association of ligand to LBD, CAR is released from heat shock receptor (HSP90), translocated from cytosol to nucleus, and activated by forming a complex with the retinoid X receptor (RXR), another member of the nuclear receptor superfamily, and nuclear coactivator proteins. The activated CAR binds to a xenobiotic response element (XRE) located in the promoter region of the target genes coding CYP family proteins (CYPs).

The constitutive active/androstane receptor (CAR) is a nuclear receptor that functions as a xenobiotic sensor, which regulates the expression of enzymes involved in drug metabolism and of efflux transporters. Evaluation of the binding properties between CAR and a drug was assumed to facilitate the prediction of drug–drug interaction, thereby contributing to drug discovery. The purpose of this study is to construct a system for the rapid evaluation of interactions between CAR and drugs. We prepared recombinant CAR protein using the Escherichia coli expression system. Since isolated CAR protein is known to be unstable, we designed a fusion protein with the CAR binding sequence of the nuclear receptor coactivator 1 (NCOA1), which was expressed as a fusion protein with maltose binding protein (MBP), and purified it by several chromatography steps. The thus-obtained CAR/NCOA1 tethered protein (CAR-NCOA1) was used to evaluate the interactions of CAR with agonists and inverse agonists by a thermal denaturation experiment using differential scanning fluorometry (DSF) in the presence and absence of drugs. An increase in the melting temperature was observed with the addition of the drugs, confirming the direct interaction between them and CAR. DSF is easy to set up and compatible with multiwell plate devices (such as 96-well plates). The use of DSF and the CAR-NCOA1 fusion protein together allows for the rapid evaluation of the interaction between a drug and CAR, and is thereby considered to be useful in drug discovery.

Key words constitutive androstane receptor; pregnane X receptor; nuclear receptor
tor 1 (NCOA1), which was expressed as a fusion protein with maltose binding protein (MBP) and purified by several chromatography steps. The thus obtained CAR/NCOA1 tethered protein (CAR-NCOA1) exhibited a melting curve, and an increase in the melting temperature was observed with the addition of the drugs. It could be assumed that the use of DSF and CAR-NCOA1 fusion protein together allows for the rapid evaluation of the interaction between a drug and CAR, and thus would contribute to drug discovery.

MATERIALS AND METHODS

Construction of Expression Vectors for MBP and MBP-CAR-NCOA1 The DNA fragment coding MBP was amplified by PCR with the primers GCTCTAGAGCATATGAAA CTG AAG AAG GTA AAC and AGACTGAGATTAGTCTGGCGC GTCTTTTCCG from pMAL (NEB) as a template and was digested using NdeI and XhoI. The digested DNA fragment was cloned into pET-22b (+) (Novagen) using the same sites. The resultant plasmid was named pET-MBP-His. The DNA fragment coding the fusion protein between the LBD of the constitutive androstane receptor (CAR) and the NCOA1-derived sequence was synthesized (Integrated DNA Technologies) and cloned into pCold-MK, a modified version of pCold-I and was digested using NdeI and XhoI. The digested DNA fragment was cloned into pET-22b (Novagen) using the same sites. The resultant plasmid was named pET-MBP-His. The DNA fragment coding the fusion protein between the LBD of CAR and the C-terminus hexa-histidine tag. The resultant construct was as follows: MBP–HRV3C protease digestion site–LBD of CAR–CAR binding sequence of NCOA1–hexa-histidine tag. The resultant plasmid was named pCold-MK-CAR-NCOA1-His.

Preparation of Gelatinized Starch Corn starch powder (0.1 g; Wako) was suspended in 1 mL of water in a 1.5 mL or 2.0 mL microtube at room temperature. The corn starch suspension was heated at 95 °C for 10 min with occasional agitation by using a vortex mixer, which was centrifuged for 2 min at 12000 rpm. The supernatant was removed by pipetting, after which about 1 mL of water was added to the precipitate for agitation using the vortex mixer, followed by centrifugation for 2 min at 12000 rpm. This process was conducted five times. After washing in water five times, the gelatinized starch resin was equilibrated by repeated washing with buffer A (50 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 150 mM NaCl, pH 7.4). The equilibrated resin could be stored at 4 °C for several days; with longer storage times, a large visible aggregate of starch formed.

Expression and Purification of the MBP-Tagged CAR-NCOA1 Fusion Protein Escherichia coli BL21(DE3) competent cells were transformed with both pCold-MK-CAR-NCOA1-His and chaperone plasmid pG-KJE8 (TaKaRa, Shiga, Japan). A single colony was inoculated into 30 mL of TB medium containing both 50 µg/mL kanamycin and 50 µg/mL chloramphenicol and incubated at 37 °C overnight with constant shaking (approx. 150 rpm). Cells were collected by centrifugation at 4 °C for 5 min at 5000 rpm, resuspended in 300 mL of TB medium containing 50 µg/mL kanamycin, 50 µg/mL chloramphenicol, and 5 ng/mL tetracycline, and incubated at 37 °C with constant shaking (approx. 150 rpm) until the OD600 reached 2.5–3.0, after which it was cooled in an ice bath to 15 °C. Expression of the recombinant proteins was induced by adding IPTG to a final concentration of 0.4 mM. The culture was further incubated at 15 °C for 48 h with gentle agitation (approx. 110 rpm). The cells were harvested by centrifugation at 4000 × g at 4 °C for 15 min and were resuspended in 50 mM HEPES (pH 7.4), 150 mM NaCl buffer and disrupted by sonication. MBP-CAR-NCOA1 was purified by gelatinized starch prepared from 1 g of raw starch. Cell lysate was mixed with the gelatinized starch and gently rotated for about 1 h at 4 °C, followed by centrifugation at 4000 × g and 4 °C for 5 min, removal of the supernatant, and resuspension of the precipitate into 30 mL of 50 mM HEPES (pH 7.4), 150 mM NaCl buffer. This washing step to remove unbound protein was conducted three times, after which the precipitate was resuspended in 20 mL of 50 mM HEPES (pH 7.4), 150 mM NaCl buffer containing 10 mM maltose and gently rotated for 30 min at 4 °C to elute the MBP-fusion protein. The gelatinized starch was removed by centrifugation at 4000 × g at 4 °C for 5 min, and the supernatant was collected in another tube. To this eluent, HRV3C protease was added to remove MBP-tag and reacted overnight, followed by further affinity purification by Ni-NTA and gel filtration chromatography using HiLoad 16/600 Superdex 75 pg prep grade (GE Healthcare Biosciences, Chicago, IL, U.S.A.) with a running buffer of 50 mM HEPES (pH 7.4), 150 mM NaCl.

Differential Scanning Fluorometry Measurements Differential scanning fluorometry measurements were car-

Fig. 1. Design of Construct for Production of CAR-NCOA1 Fusion Protein
The N-terminus MBP was followed by the HRV3C protease digestion site, CAR-NCOA1 fusion, and the C-terminus hexahistidine tag.
ried out as described previously with CFX connect (Bio-Rad, Hercules, CA, U.S.A.). All scans were obtained at a protein concentration of 2 µM and ligand concentrations of 0, 1, 2, 4, 10, and 20 µM. All measurements were acquired in 50 mM HEPES, 150 mM NaCl buffer (pH 7.4) containing 5% dimethyl sulfoxide (DMSO) and SYPRO Orange Protein Gel Stain (ThermoFisher, Waltham, MA, U.S.A.) diluted 1000 times. The samples were incubated at 37°C for 30 min immediately before DSF measurements. All DSF scans were measured from 25 to 80°C at a scanning rate of 1.0°C/min.

RESULTS

Design of CAR-NCOA1 Fusion Protein It was reported that an artificial protein, in which a flexible peptide linker tethered the LBD binding sequence derived from NCOA1 to the LBD of pregnane X receptor (PXR; the other member of the nuclear receptor family that is also related to drug metabolism), exhibited markedly improved stability and yield for biophysical studies..

We therefore designed the CAR-NCOA1 fusion protein based on both of these reports and the crystal structure of the CAR-NCOA1 complex13 (Fig. 1, Fig. S1). The N-terminus MBP was followed by the HRV3C protease digestion site, CAR-NCOA1 fusion, and the C-terminus hexahistidine tag. MBP is an extensively used affinity tag that associates amylose. Moreover, fusion with MBP has been shown to enhance the solubility of proteins expressed in E. coli.15–17 This construct was used to prepare CAR-NCOA1 fusion protein.

Expression and Purification of CAR-NCOR1 Fusion Protein by Using Gelatinized Starch The above-designed construct was expressed in E. coli and purified by several chromatography steps. Affinity chromatography medium is a chemically cross-linked starch that is frequently employed for the purification of MBP fusions.18,19 A combined agarose-amylose bead for chromatography is commercially available, though expensive (33000 JPY/15 mL with a net resin volume of about 10 mL). Moreover, the immobilized amylose on the beads is degraded by amylase derived from E. coli, which decreases the binding capacity for re-use.20 Starch is a polysaccharide composed of 20 to 25% amylose and 75 to 80% amylopectin by weight.21 Reportedly, raw starch could be used to purify MBP fusion proteins.22 In the present study, corn starch was used as a chromatography medium instead of commercially available amylose resin. Corn starch is inexpensive (2000 yen/500 g) and thus can be considered disposable. We first examined the interaction between MBP and raw starch, and found that the binding capacity was low for the preparation of proteins on the order of milligrams for biophysical studies (Fig. S2). The surface area of corn starch can be increased by gelatinization, which is assumed to increase the binding capacity of corn starch to MBP.23–28 We prepared gelatinized starch by heating it in excess water and then repeatedly washing it in cold water and buffer solution. This gelatinization significantly improved the binding amount of corn starch to MBP. Gelatinized starch was therefore used as the affinity purification medium for the preparation of MBP-CAR-NCOA1.

The MBP-CAR-NCOA1 fusion protein was expressed in the E. coli BL-21(DE3) strain. The harvested cells were disrupted by sonication and centrifuged to remove cell debris, after which lysate was collected. Gelatinized starch was added to the lysate and washed three times to remove unbound protein. The MBP-CAR-NCOA1 fusion protein was eluted by using buffer containing maltose, followed by MBP removal by HRV3C protease digestion, affinity chromatography using Ni-NTA, and gel filtration chromatography (Fig. 2). Two bands were observed in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the eluent of the gelatinized starch. The major band was derived from the MBP-CAR-NCOA1 fusion, since this band was separated into two by HRV3C protease digestion. One of the two bands was derived from the CAR-NCOA1 fusion and the other from the digested MBP. Affinity purification by Ni-NTA resulted in a single band derived from the CAR-NCOA1 fusion. Finally, about 7.5 mg of the CAR-NCOA1 fusion protein was obtained from 300 mL of terrific broth (TB) culture medium, which was enough for biophysical and structural studies.

DSF Analysis of CAR-NCOA1 Fusion Protein We performed DSF measurements of CAR-NCOA1 at different concentrations of three inverse agonists, clotrimazole (Figs. 3A–C), PK-11195 (Figs. 3A, D, E), and T0901317 (Figs. 3A, F, G). DSF analysis was also performed for an agonist, CITCO29,30 (Figs. 3A, H, I). As a negative control experiment, DSF measurements at different SR12813 concentrations were also performed (Figs. 3J, K).
fluorescence intensity was high at the lower temperature, then decreased in a sigmoidal manner as the temperature increased. The DSF curves of typical proteins obey a sigmoid curve that rises to the right. The DSF curve of the ligand-free CAR-NCOA1 was the opposite of that. If the ligand that associated with the protein exists in the sample, the DSF curve shifts to a higher temperature. At higher ligand concentrations, the DSF curve of CAR-NCOA1 became a sigmoid curve rising to

Fig. 3. DSF Analysis of Interaction between CAR-NCOR1 Fusion Protein and Drugs

(A) Structure of clotrimazole, PK-11195, T0901317, CITCO and SR-12813. Three compounds, clotrimazole, PK-11195, and T0901317, are inverse agonists. CITCO is an agonist. SR-12813 is neither a CAR agonist nor inverse agonist, thereby used as a negative control. (B) The DSF curves at 2 µM of the CAR-NCOA1 fusion protein at different concentrations of clotrimazole (B), PK-11195 (D), T0901317 (F), CITCO (H) and SR-12813 (J). The derivatives of the fluorescence intensities (dF/dT) plotted against temperature at 2 µM of the CAR-NCOA1 fusion protein at different concentrations of clotrimazole (C), PK-11195 (E), T0901317 (G), CITCO (I) and SR-12813 (J). The drug concentrations were 0 µM (red open triangle), 1 µM (blue closed triangle), 2 µM (green open circle), 4 µM (black closed circle), 10 µM (magenta open rectangle), and 20 µM (light blue closed rectangle), respectively. (Color figure can be accessed in the online version.)
the right and $T_m$ (denaturing midpoint) shifted depending on the ligand concentration. These results suggested that SYPRO orange dye associated with the ligand-binding pocket of CAR-NCOA1 and competed with clotrimazole, PK-11195, T0901317, and CITCO. As the ligand concentration increased, the ligand-binding pocket of CAR-NCOA1 was replaced from SYPRO orange dye to clotrimazole, PK-11195, T0901317, or CITCO, which resulted in a change in the shape of the DSF curve to those typical of proteins. We reported a similar observation for another nuclear receptor, PXR. In contrast to the case with clotrimazole, PK-11195, T0901317, and CITCO, the DSF curve did not change when the concentration of SR12813 increased increased (Figs. 3E, F). It could be assumed that the CAR-NCOA1 protein allows for the detection of an association between the ligand and the drug binding pocket of CAR protein.

The $\Delta T_m$ values at a ligand concentration of 20 $\mu$M are listed in Table 1. Among the three inverse agonists, clotrimazole exhibited the highest $\Delta T_m$ value. Previous time-resolved FRET (TR-FRET) analysis revealed that clotrimazole is a stronger inverse agonist for CAR than PK-11195. It was also reported that PK-11195 was a stronger inverse agonist than T0901317. The highest $\Delta T_m$ value of clotrimazole among the three tested inverse agonists was assumed to reflect the highest affinity of clotrimazole for CAR. The agonist, CITCO, exhibited the highest $\Delta T_m$ value among the five ligands tested. Association of the agonist to the LBD region of CAR causes a structural rearrangement of the C-terminus helix, helix 12, which makes contact with NCOA1. On the other hand, helix 12 is not in contact with NCOA1 and is disordered in the inverse-agonist bound state (Fig. S3). It was therefore assumed that the melting temperature was higher in the agonist-bound state than in the inverse agonist-bound state. Comparison of $\Delta T_m$ values among agonists or among inverse agonists was assumed to allow for the determination of affinity, while comparison between agonists and antagonists required attention. That is, although CAR-NCOA1 fusion protein can be used to rapidly search for a binding ligand, another experiment was required to distinguish an agonist from an inverse agonist.

**DISCUSSION**

In this paper, we described that the fusion protein between CAR LBD and its binding sequence derived from NCOA1 was a stable construct for production in an *E. coli* expression system. We also showed that the combination of CAR-NCOA1 fusion protein and DSF could be used to rapidly evaluate the binding between CAR LBD and ligands. CAR agonist induces the expression of CYP proteins and causes drug–drug interactions. CAR is also involved in various hepatic functions.

**Table 1. Summary of $\Delta T_m$ of CAR-NCOA1 Fusion Protein at the Ligand Concentration of 20 $\mu$M**

| Ligand  | $\Delta T_m$ (°C) |
|---------|------------------|
| Clotrimazole | 9.1 (+/−0.2) |
| CITCO   | 12.7 (+/−0.3) |
| PK11195 | 5.9 (+/−0.2) |
| T0901317| 2.4 (+/−0.5) |
| SR-12813| −0.6 (+/−0.1) |

Besides drug–drug interactions, food may influence pharmacokinetics. For example, hyperforin, contained in St. John’s wort, is a strong agonist of PXR and affects drug metabolism. The ligand specificity of LBDs from CAR and PXR is low, and many drugs associate to them. We previously reported that PXR-NCOA1 could be used to evaluate interactions between ligand and PXR. The combined use of fusion proteins, PXR-NCOA1 and CAR-NCOA1, will afford information about drug–drug interactions. The present results would contribute to drug discovery in the future.

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**Author Contributions** YK and MN contributed equally to the present work. YK and HM designed the experiment. YK, TS, MS, and HM wrote the paper. YK, MN, YI, SY, KO, YT, TS, MS, and HM performed the experiment and analyzed the data. All authors discussed the results.

**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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