Feasibility of Structural Modification of Retinoid X Receptor Agonists to Separate Blood Glucose-Lowering Action from Adverse Effects: Studies in KKA\(^{a}\) Type 2 Diabetes Model Mice

Hiroki Kakuta,*\(^{a}\) Fuminori Ohsawa,\(^{a}\) Shoya Yamada,\(^{a}\) Makoto Makishima,\(^{b}\) Akihiro Tai,\(^{c}\) Hiroyuki Yasui,\(^{d}\) and Yutaka Yoshikawa\(^{d}\)

\(^{a}\) Division of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences; 1–1–1 Tsushima-Naka, Okayama 700–8530, Japan; \(^{b}\) Division of Biochemistry, Department of Biomedical Sciences, Nihon University School of Medicine; 30–1 Oyaguchi-kamicho, Itabashi-ku, Tokyo 173–8610, Japan; \(^{c}\) Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima; Shobara, Hiroshima 727–0023, Japan; and \(^{d}\) Kyoto Pharmaceutical University; 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607–8414, Japan. Received November 11, 2011; accepted January 4, 2012

Retinoid X receptors (RXRs) function as heterodimers with peroxisome proliferator-activated receptors (PPARs), which are the target of thiazolidinediones used to treat type 2 diabetes,\(^{1)}\) and also with liver X receptors (LXRs), which are reported to show hypoglycemic action when activated.\(^{2)}\) Since PPAR/RXR or LXR/RXR heterodimers can be activated even by RXR agonists alone (permissive mechanism),\(^{3)}\) RXR agonists are considered to be candidates for the treatment of type 2 diabetes.\(^{4)}\) Although the hypoglycemic action of RXR agonists is thought to be mediated by PPAR/RXR and LXR/RXR activation, it is not known which heterodimer is predominantly involved. On the other hand, RXR agonists have also been reported to induce hepatomegaly,\(^{5)}\) blood triglyceride (TG) elevation\(^{6)}\) and other adverse effects. Among them, the TG elevation by RXR agonists is mainly caused by LXR/RXR activation via the permissive mechanism.\(^{7)}\)

Mogami et al. reported that structurally different RXR agonists show different patterns of activation of RXR-heterodimers.\(^{8)}\) These findings motivated us to create structurally modified RXR agonists (NEt-TMN)\(^{9)}\) and NEt-3IB\(^{10)}\) (Fig. 1A). We found that these RXR agonists similarly activate RXR, but differently activate PPAR/RXR and LXR/RXR.\(^{11)}\) Thus, in order to examine the feasibility of separating the blood glucose-lowering action of RXR agonists from the adverse effects, we administered the RXR agonists NEt-TMN, NEt-3IB, and NEt-3IP to KKA\(^{a}\) type 2 diabetes model mice, and examined the relationship of RXR-heterodimer activation pattern to antihyperglycemic effect and adverse effects such as hepatomegaly and TG elevation.

Retinoid X receptor (RXR) agonists are reported to exhibit blood glucose-lowering action owing to peroxisome proliferator-activated receptor (PPAR)/RXR or liver X receptor (LXR)/RXR activation, but may also cause adverse effects such as blood triglyceride elevation. In order to examine the feasibility of separating the glucose-lowering action from the adverse effects, we examined the effects of RXR agonists (NEt-TMN), NEt-3IB, and NEt-3IP, which have different heterodimer-activating patterns, in KKA\(^{a}\) type 2 diabetes model mice. We found that NEt-3IB induced lower degrees of hepatomegaly and blood triglyceride (TG) elevation than the other RXR agonists, even though all of them showed similar blood glucose-lowering action on repeated administration. These findings indicate that structural modification of RXR agonists is a potentially effective strategy to reduce adverse effects while retaining desired activities.

Key words retinoid X receptor; type 2 diabetes; glucose-lowering action; adverse effect; hepatomegaly; triglyceride elevation

Fig. 1. Chemical Structures of RXR Agonists NEt-TMN, NEt-3IB and NEt-3IP, and Results of Reporter Gene Assay

(A) Chemical structures of NEt-TMN, NEt-3IB and NEt-3IP. (B) Relative transactivation activity, based on the luciferase activity of 1 μM LGD1069 taken as 1.0, toward RXRα. Significant differences: **p<0.01 vs. NEt-TMN.

*To whom correspondence should be addressed. e-mail: kakuta@pharm.okayama-u.ac.jp © 2012 The Pharmaceutical Society of Japan
MATERIALS AND METHODS

Chemicals NEt-TMN was prepared according to ref. 9. NEt-3IB and NEt-3IP were prepared according to ref. 10. TIPP703 (PPAR-pan agonist) and carba-T0901317 (LXR agonist) were kindly provided by Dr. Miyachi (Okayama University).

Culture of COS-1 Cells COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ at 37°C.

 Luciferase Reporter Gene Assay Luciferase reporter gene assays were performed using COS-1 cells transfected with three kinds of vectors encoding a receptor, luciferase reporter gene under the control of the appropriate response elements, and secreted alkaline phosphatase (SEAP) gene. CRBPII-tk-Luc, tk-PPREx3-Luc, and tk-tBARx3-Luc contain RXR, PPAR, and LXR response elements, respectively. The amounts of receptor and response element were 1.0 and 4.0 μg, respectively. Transfection was performed with QIA Effectene Transfection reagent according to the supplier’s protocol. In the case of heterodimer assay, RXRα (0.5 μg), the partner receptor (PPARγ or LXRα, 0.5 μg) and the partner response element (4.0 μg) were transfected into COS-1 cells as described above.

Compound solutions in which the dimethyl sulfoxide (DMSO) concentration was below 1% were added to a suspension of transfected cells seeded at about 2.0×10⁵ cells/well in 96-well white plates. After incubation in a humidified atmosphere of 5% CO₂ at 37°C for 18 h, 25 μL of the medium was used for analyzing SEAP activity and the remaining cells were used for luciferase reporter gene assay with a Steady-Glo Luciferase Assay system (Promega) according to the supplier’s protocol. The luciferase activities were normalized using the SEAP activities. The assays were carried out in triplicate three times.

KKA⁺ Mice Six-week-old male KKA⁺ mice were purchased from CLEA Japan, Inc. (Tokyo, Japan), individually housed in plastic cages, and maintained on a 12-h light/dark cycle in our temperature-controlled central animal facility. All mice were allowed ad libitum access to solid food (MF, Oriental Yeast Co., Tokyo, Japan) and tap water. KKA⁺ mice were randomly divided into compound-treated and untreated groups. NEt-TMN, NEt-3IB, NEt-3IP or vehicle alone (polyethylene glycol 400) was administered daily to the mice by oral gavage for 2 weeks, starting from 10 weeks of age. Body weight was measured daily at 10:00 a.m., and then each compound was administered per os (p.o.) at 10 mg/kg/d. The KKA⁺ mouse experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and were performed according to the Guidelines for Animal Experimentation at KPU.

Glucose Level in Blood Samples for baseline measurements of fed blood glucose level were taken daily at 11:00 a.m. from the tail vein of the mice, and glucose was measured by using the glucose oxidase method (Glucocard, Arkray, Kyoto, Japan).

Blood Sampling and Assay Blood samples were collected at the day of the final administration of each compound (day 14). Mice were exsanguinated under ether anesthesia with heparinized tools, and samples were centrifuged at 3000 rpm for 10 min at 4°C. The resultant plasma was used for the analyses of biochemical parameters. Plasma adiponectin level was measured using enzyme-linked immunosorbent assay (ELISA) kits (Quantikine®) purchased from R&D Systems Inc. (Minneapolis, U.S.A.). Plasma insulin level was measured using a Lebis insulin-mice (T type) ELISA KIT (Shibayagi Co., Ltd., Gunma, Japan). HbA1c level in blood obtained from the tail vein of the mice was measured by using a DCA 2000 system (Bayer Medical Co., Tokyo, Japan). Plasma leptin level was measured using ELISA kits (Quantikine®) purchased from R&D Systems Inc. (Minneapolis, U.S.A.).

Plasma Triglyceride (TG) and Total Cholesterol (TCHO) Levels TG and TCHO levels were obtained by using a Fuji Dry Chem analyzer (Fuji Medical Co., Ltd., Tokyo, Japan).

Liver Weight After exsanguination, the liver of each mouse was removed immediately and weighed.

Statistical Analysis All experimental results are expressed as the mean values±standard error of the mean (S.E.M.). Statistical analysis was performed by analysis of variance (ANOVA), and differences were considered significant at the 1% or 5% probability level.

RESULTS

Figures 1 and 2 show the chemical structures of three different RXR agonists NEt-TMN, NEt-3IB and NEt-3IP and the reporter-gene data obtained for RXRα, PPARγ, LXRα, PPARγ/ RXRα, and LXRα/RXRα. The data for NEt-TMN and NEt-3IB were taken from ref. 11. NEt-TMN and NEt-3IB show similar levels of RXR activation, while NEt-3IP was less potent, activating RXRα in the concentration range between 10⁻⁸ M and 10⁻⁷ M (Fig. 1B). Although they showed no PPAR agonistic activities (Fig. 2A), all three compounds activated PPAR/RXR. Among them, NEt-3IP was more effective than NEt-3IB, and as effective as NEt-TMN in the concentration range between 10⁻⁷ M and 10⁻⁶ M (Fig. 2B). As for LXR and LXR/RXR activities, NEt-TMN was the most potent, while NEt-3IB and NEt-3IP showed similar potencies (Figs. 2C, D).

Figure 3A shows changes in blood glucose concentration in KKA⁺ mice when each compound was administered at 10 mg/kg/d p.o. for 14 d. While the average blood glucose level in vehicle-treated mice was about 500 mg/dL, the levels in NEt-TMN- and NEt-3IP-treated mice were about 300 mg/dL from day 3 after the start of administration, showing a significant blood glucose-lowering effect. NEt-3IB also showed apparent efficacy, but not until day 5, exhibiting a longer lag time than NEt-TMN and NEt-3IP. All compounds decreased the blood glucose level to about 300 mg/kg from 5 d after the beginning of the treatment, and this was significantly lower than the value in animals treated with the vehicle alone. On the other hand, administration of these compounds induced weight gain compared with the vehicle controls. In particular, NEt-3IP induced a clear weight increase by day 7 (Fig. 3B).

HbA1c, which reflects long-term glycemic control, is shown in Fig. 4A. Compared with the vehicle, the administration of each compound apparently reduced HbA1c from 10 to 7–8%. The concentration of adiponectin, which is associated with insulin resistance, was in order of NEt-3IP<NEt-3IB<NEt-TMN (Fig. 4B). Each compound exhibited a tendency to reduce insulin concentration, though without statistical significance (Fig. 4C). RXR agonists are reported to induce hepatomegaly or blood triglyceride elevation and these phenomena...
are related to LXR/RXR activation. Since the RXR agonists used in this research possess different LXR/RXR activating potentials, we were interested in the occurrence of adverse effects arising from LXR/RXR activation. While NEt-TMN or NEt-3IP treatment increased liver weight up to about 2.6 g (vehicle alone: 1.6 g), NEt-3IB treatment increased it to only 2.1 g (Fig. 4D). As for blood total cholesterol (TCHO), NEt-TMN, NEt-3IB, and NEt-3IP induced increases of 1.4-, 1.5- and 1.6-fold, respectively, as compared to the vehicle controls (Fig. 4E). Blood triglyceride level (TG) was also increased by NEt-TMN, NEt-3IB, and NEt-3IP treatment by 1.6-, 1.5- and 1.8-fold, respectively, relative to the vehicle controls (Fig. 4F).

**DISCUSSION**

It has been reported that NEt-TMN and NEt-3IB possess similar RXR agonistic activation characteristics, but NEt-TMN is more a potent activator than NEt-3IB for PPAR/RXR and LXR/RXR. NEt-3IP is a less potent RXR agonist than NEt-TMN and NEt-3IB. Here, we found that the PPAR/RXR transactivation efficacy of the three compounds was in the order of NEt-TMN ≈ NEt-3IP > NEt-3IB in the range 10^{-7} to 10^{-5} M, and the LXR/RXR activation activity was in order of NEt-TMN > NEt-3IB > NEt-3IP. Since these compounds are detected in the concentration range above 1 μM when orally administered at 30 mg/kg (data not shown), to examine the
physiological implications of these differences, NEt-TMN, NEt-3IB and NEt-3IP were each administered to KKA\textsuperscript{2} diabetes model mice, and the relationship between RXR-heterodimer activation pattern and antihyperglycemic effect or adverse effects such as hepatomegaly or TG elevation was assessed.

NEt-TMN and NEt-3IP lowered blood glucose level from 500 mg/dL to about 300 mg/dL soon after the administration. Despite having less potent RXR and LXR/RXR activities than NEt-TMN, NEt-3IP showed similar PPAR/RXR activation to NEt-TMN. The blood glucose-lowering action of NEt-3IB was slower, and the PPAR/RXR activity of NEt-3IB was less potent than that of the other compounds, indicating that blood glucose-lowering action by RXR agonists may be mainly related to PPAR/RXR activation. Since each compound had blood glucose-lowering activity, we next examined the secretion of adiponectin, which is known to be associated with improvement of insulin resistance, and insulin concentration. Each compound increased adiponectin secretion and slightly decreased insulin concentration. The magnitudes of the changes in the concentrations of adiponectin and insulin in blood were in order of NEt-TMN, NEt-3IB, NEt-3IP, which corresponds not to activation of PPAR/RXR, but to activation of LXR/RXR. In terms of blood glucose level on day 14, all compounds showed similar blood glucose-lowering action, and adiponectin secretion is reported to be regulated by not only PPAR/RXR,\textsuperscript{13} but also LXR/RXR,\textsuperscript{16} implying that early blood glucose lowering may be mediated by PPAR/RXR, and later blood glucose lowering and adiponectin secretion may be mediated by LXR/RXR.

Each compound increased body weight compared with vehicle treatment. In particular, NEt-3IP induced a significant weight gain on day 7 after the start of administration. All compounds induced elevation of blood TG and TCHO, and no correlation between these adverse effects and RXR-heterodimer activation could be found. However, although repeated administration of each compound produced similar blood glucose-lowering action, NEt-3IP induced the greatest elevation of blood TG and TCHO. From the viewpoint of the balance between beneficial and adverse effects, it is interesting that NEt-3IB induced lower levels of hepatomegaly and TG elevation compared to the other compounds, but still retained the blood glucose-lowering action after repeated administration. This result indicates that it may be feasible to structurally modify RXR agonists in ways that would retain the desired blood glucose-lowering action while reducing the severity of side effects.

In conclusion, NEt-3IB was found to induce lower degrees of hepatomegaly and TG elevation and maintained its blood glucose-lowering action after repeated administration, though the onset of blood glucose lowering by NEt-3IB was delayed compared to the other two compounds. These results indicate that structural modification of RXR agonists may be an effective strategy to separate the activities, and this approach may yield clinically useful RXR agonists with reduced side effects.

Acknowledgments This research was financially supported by JST (Adaptable and Seamless Technology Transfer Program through Target-driven R&D), and Takeda Science Foundation. We also thank Dr. Miyachi (Okayama University) for kindly providing TIPP703 and carba-T0901317. The authors are also grateful to Professor Yoshio Naomoto and Dr. Takuya Fukazawa (Department of General Surgery, Kawasaki Medical School) for preparing plasmids.

Fig. 4. HbA1c (A), Serum Adiponectin (B), Insulin (C), TCHO (E) and TG (F) Levels and Liver Weight (D) of Control Diabetic KKA\textsuperscript{2} Mice and KKA\textsuperscript{2} Mice Treated with NEt-TMN, NEt-3IB and NEt-3IP

Blood samples were collected at the day of the final compound administration (day 14). n=5–7. Significant differences: \(*p<0.05\) vs. vehicle. **\(p<0.01\) vs. vehicle.
REFERENCES


