Research article

Identification and characterization of a phenyl-thiazolyl-benzoic acid derivative as a novel RAR/RXR agonist

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ABSTRACT

Objective: To identify an agonist of RXRα and RARα with reduced undesired profiles of all-trans retinoic acid for differentiation-inducing therapy of acute promyelocytic leukemia (APL), such as its susceptibility to P450 enzymes, induction of P450 enzyme, increased sequestration by cellular retinoic acid binding protein and increased expression of P-glycoprotein, a virtual screening was performed.

Results and conclusion: In this study, a phenyl-thiazolyl-benzoic acid derivative (PTB) was identified as a potent agonist of RXRα and RARα. PTB was characterized in nuclear receptor binding, reporter gene, cell differentiation and cell growth assays. PTB bound directly to RXRα and RARα, but not to PPARα, δ or γ. PTB fully activated reporter genes with enhancer elements for RXRα/RXRα and partially activated reporter genes with enhancer elements for RARα/RXRα, PPARα(β) and PPARγ. Furthermore, PTB induced differentiation and inhibited the growth of human APL cells. Thus, PTB is a novel dual agonist of RXRα and RARα and works as both a differentiation inducer and a proliferation inhibitor to leukemic cells.

1. Introduction

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia with the t(15; 17) (q22; q21) chromosomal translocation which involves promyelocytic leukemia (PML) and retinoic acid receptor alpha (RARα) genes and produces the two chimeric fusion proteins, PML-RARα and reciprocal RARα-PML [1, 2]. The PML-RARα fusion protein exhibits dominant negative effects on both PML and RARα pathways, prevents promyelocytes maturation and then leads to immature leukemic cells accumulation [3]. Moreover, PML fused to RARα transforms a RAR-retinoid X receptor (RXR) heterodimer into an oncogenic PML-RARα homodimer, and this enforced RARα homo-dimerization is considered as a common mechanism to block transcription and differentiation by various RARα fusion proteins [4].

In the late 1980s, the all-trans retinoic acid (ATRA)-based therapy, which induces hematological complete remission (CR) in APL patients [5], has dramatically advanced the treatment of APL. The ATRA-based therapy, initially classified as a differentiation therapy, is now regarded as a molecular-targeted therapy aimed at the pathogenic PML-RARα [6]. Although ATRA has the beneficial effect on APL [7, 8, 9], an average duration of the hematological CR with ATRA is several months [10], and in some cases before reaching CR, APL acquires resistance against ATRA and then relapses within a short period [11]. There are a few mechanisms believed to explain the ATRA resistance [12, 13]. First, a continuous ATRA treatment causes a progressive reduction in plasma drug concentration, partly by increasing drug metabolism due to the induction of cytochrome P450 enzymes [14, 15, 16]. Second, increased levels of cellular retinoic acid binding protein (CRABP) in ATRA-resistant leukemic cells prevents ATRA to enter enough into the nucleus [17, 18]. Third, ATRA might be eliminated by P-glycoprotein, which is a transmembrane drug efflux pump involved in resistance to multiple chemotherapeutic agents and is increased in ATRA-resistant leukemic cells [16]. Furthermore, a missense mutation in RARα region of PML-RARα fusion gene has been identified in the APL cells of relapsed patients. The mutation located in the ligand-binding domain of RARα prevents the interaction of PML-RARα with ATRA and reverses the effect of ATRA on myeloid differentiation [19].

RAR and RXR forms a heterodimer which plays important roles in...
Fig. 1. A: The structure of 4-(4-(3-trifluoromethyl-phenyl)-thiazol-2-yl)-benzoic acid derivative (PTB). B: Modeled structure of PTB (atom color) in the ligand binding pocket of RXRα. 9-cis RA (magenta) is overlaid as a reference. C-G: The receptor profiles of PTB by TR-FRET binding assay. Values are expressed by mean ± s.e.m. (n = 3). H-K: The receptor profile of PTB by reporter gene assay. Values are expressed by mean ± s.e.m. (n = 3).
myelocyte differentiation and apoptosis, and the PML-RARα fusion protein represses RAR/RXR signaling pathway [4]. In HL-60 cells that does not carry the typical translocation but has a capacity to differentiate, ligand-induced RARα activation is enough to induce differentiation, whereas RXRα activation could induce apoptosis by downregulating Bcl-2 mRNA [20, 21]. Moreover, a combination of RXR and RARα ligands could enhance differentiation synergistically in differentiation-resistant APL cell line [22].

In this study, a virtual screening was performed to identify an agonist of RXRα and RARα with reduced undesired profiles of ATRA for the treatment of APL, and a phenyl-thiazolyl-benzoic acid derivative (PTB; Key Organics Limited, Catalog No. 1G-433S) as shown in Fig. 1A was identified as one of the most promising compounds since it showed very good overlap with a known agonist 9-cis RA, and had excellent complementarity to the binding site as depicted in Fig. 1B.

2. Results

2.1. Virtual screening

Virtual screening of a commercial database against the agonist-bound form of RXRα was performed using the docking program GLIDE (Schrödinger, LLC, New York, NY) and refined parameters. Through a post-docking analysis involving a visual inspection, a phenyl-thiazolyl-benzoic acid derivative (PTB; Key Organics Limited, Catalog No. 1G-433S) as shown in Fig. 1A was identified as one of the most promising compounds since it showed very good overlap with a known agonist 9-cis RA, and had excellent complementarity to the binding site as depicted in Fig. 1B.

2.2. The receptor selectivity profiles

Direct binding of PTB to RXRα and RARα was evaluated by using TR-FRET assay. PTB showed agonistic activities for both RXRα and RARα (Fig. 1C–D). Direct binding of PTB to any of PPARs was not observed (Fig. 1E–G). PTB has the highest affinity for RXRα among the nuclear receptors tested. EC50 values of PTB to several nuclear receptors are shown in Table 1.

The reporter gene assays were carried out to examine functional effects of PTB in cellular systems. The nuclear receptors, RXRα/RXRα, RARα/RXRα, PPARα/PPARγ, PPARβ/PPARδ, and PPARγ/PPARδ, were tested in reporter gene assays. The results are shown in Fig. 1H–K. PTB fully activated RXRα/RXRα reporter gene, and partially activated RARα/RXRα, PPARα/PPARγ, and PPARγ/PPARδ reporter genes. EC50 values were not able to be calculated in PPARδ/PPARγ and PPARγ/PPARδ reporter genes because the signals did not reach plateau in PPARα/PPARγ and PPARγ/PPARδ reporter genes. The obtained EC50 values and fold increase in activation at 10 μM are listed in Table 1.

2.3. Effect of PTB on differentiation of APL cell lines

It is well known that RAR agonists induce the differentiation of APL cells. Therefore, the effect of PTB on RAR mediated induction of differentiation in NB4 cells was examined. PTB-treated NB4 cells induced tetrazolium reduction ability, an indicator of differentiation, in a dose dependent manner (Fig. 2A). Calculated EC50 value of PTB to induce differentiation based on the reduction activity of tetrazolium salt was 0.95 μM.

To confirm the differentiation, PTB effects on CD11b expression on differentiated leukemia cell surface were examined by flow cytometry because it is known that differentiated NB4 and HL-60 cells express the CD11b antigen. PTB induced the expression of CD11b antigen in a dose dependent manner that is consistent with the tetrazolium reduction assay. The differentiation inducing profiles of PTB was almost the same between NB4 and HL-60 (Fig. 2B–C).

2.4. Anti-leukemic activity of PTB in vitro and in vivo

PTB inhibited proliferation of HL-60 cells with IC50 value of 0.71 μM (Fig. 2D), and inhibited NB4 subcutaneous tumor growth significantly by 44% at 20 mg/kg given orally once daily (Fig. 2E).

3. Discussion and conclusion

PTB was identified as a novel RXRα and RARα agonist by virtual screening. It showed a very good structural overlap with a known agonist, 9-cis RA, and had excellent complementarity to the binding site of RXR. After we identified and characterized PTB in early 2000, we noted that PTB was also reported as a candidate of RAR ligand in a literature [23], where PTB was identified as a possible RARα ligand by virtual screening, docked into the binding pocket of RAR (important residues as R274, R278 and S289) and superimposed with the crystal structure of ATRA. However, the direct binding of PTB to RARα was not investigated, although PTB activated the CAT reporter gene with RARα, RARβ, RARγ and RXRβ.

In the nuclear receptor binding assay, PTB did not bind directly to PPARα/PPARγ, but in the cellular system, PTB partially activated PPARα/PPARγ and PPARγ/PPARδ reporter genes. Activation of these reporter genes by PTB seems to be due to its activity to RXRα. ATRA also did not bind directly to PPARα/PPARγ, but activated PPARα/PPARγ and PPARγ/PPARδ reporter genes. ATRA is reported as a high affinity ligand for PPARα (β) and binds to PPARα (β) with nanomolar affinity [24]. Furthermore, they reported that ATRA did not activate PPARα reporter gene. However, their results were not reproduced and were not consistent with our results.

PTB induced differentiation of both NB4 and HL-60 cells as detected by tetrazolium reduction assay and by CD11b expression analysis. The potency of PTB for the induction of cell differentiation was similar to those of ATRA and 9-cis RA. However, the minimum concentration of PTB required to induce differentiation was higher than that of ATRA or 9-cis RA. PTB potently inhibited proliferation of HL-60 cells. The ligand activation of RAR is sufficient to induce differentiation, whereas the ligand activation of RXR is essential for the induction of apoptosis in HL-60 cells [25]. Therefore, it is suggested that the effects of PTB on

| Table 1 | EC50 [nM] values of PTB to nuclear receptors determined by binding and reporter gene assays. |
|---------|-----------------------------------------------|
| nuclear receptor binding | PTB | ATRA | 9-cis RA |
| RXRα | 454 | 175 | 35 |
| RARα | 21 | 0.36 | 0.73 |
| PPARα | No binding | No binding | No binding |
| PPARβ | No binding | No binding | No binding |
| PPARγ | No binding | No binding | No binding |
| reporter gene [fold activation at 10 μM] | RXRα/RXRα | 321 [15] | 796 [15] | 10 [12] |
| RXRα/RXRα | 86 [5.7] | 0.66 [6.8] | 2.3 [7.1] |
| PPARα/PPARγ | NA [6.3] | NA [5.2] | 121 [6.2] |
| PPARγ/PPARδ | NA [1.7] | NA [1.9] | 168 [2.4] |

NA: not applicable.
partial agonist.
induction of cell differentiation and inhibition of cell proliferation originate in its activity on RXRα and RARα.

More recently, arsenic trioxide (ATO) has been the treatment of recurrent APL, and the combination of ATRA and ATO in frontline therapy [26]. It would be also important to compare the effect of PTB to ATO and ATO/ATRA in APL models, in terms of efficacy as well as safety point of view.

In conclusion, PTB was identified as a dual agonist of RXRα and RARα.
and worked as both a differentiation inducer and a proliferation inhibitor to leukemic cells. Further characterization of PTB in patient-derived cells including ATRA-resistant cells, cellular toxicity assays, additional in vivo models, metabolic stability, pharmacokinetics and safety assessment, such as an effect on triglycerides through evaluation of LXR selectivity and SREBP1c induction, will be needed to show a possibility of its application to APL treatment.

4. Materials and methods

4.1. Nuclear receptor binding assay

PTB (Key Organics Ltd., Cat. No. 1G-433S; in 2003) was used as positive controls of the ligand binding assay to RXR, PPARα, and PPARγ. ATRA and 9-cis RA were tested for binding profiles and compared with PTB. RXRα agonist LG100268, RAR agonist TTPNB, PPARγ agonist KRP297, PPARβ agonist L-165041 and PPARy agonist BRL49653 were used as positive controls of the ligand binding assay to RXRα, RARα, PPARα, PPARβ (γ) and PPARy, respectively. TR-FRET signals from europium to allophycocyanin were measured by ARVOsx L multilabel reader. The degree of cell differentiation (net differentiation) was calculated.

4.2. Reporter gene assay

NB4 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) suspended in 100 μL of medium containing 0.01% to 10 μM of PTB, ATRA or 9-cis RA was added. Final concentration of DMSO was adjusted to 0.1%. After a 72-h incubation at 37°C, cell numbers were measured using CellTiter-Glo™ luminescent cell viability assay. Percentages of the net growth were calculated according to the following formula: 100 × (Test cell numbers – 40,000)/DMSO cell numbers – 40,000. Gl50 values were determined by non-linear curve fit analysis using GraphPad Prism.

4.3. NB4 differentiation detected by tetrazolium reduction

NB4 cells (Dainippon Pharmaceutical Co., Ltd.) were inoculated at 2 × 10^6 cells/well in 6-well plate in 2 mL of medium and 500 μL of medium containing 0.01–10 μM of PTB, 0.001–1 μM of ATRA or 9-cis RA was added. Final concentration of DMSO was adjusted to 0.1%. After a 72-h incubation at 37°C, cells were collected and stained by RPE-conjugated mouse anti-human CD11b/Mac-1 monoclonal antibody (Becton Dickinson, Cat. No. 555388) or the R-PE-conjugated mouse IgG1, κ monoclonal immunoglobulin isotype control (Becton Dickinson, Cat. No. 555749). Flow cytometry was performed by an EPICS ELITE and the results were analyzed using EPICS ELITE EXPO32 software. Differentiated cells were identified as the CD11b-positive cells.

4.4. Cell differentiation induction detected by flow cytometry

NB4 and HL-60 cells (Dainippon Pharmaceutical Co., Ltd.) were inoculated at 2 × 10^6 cells/well in 6-well plate in 2 mL of medium and 500 μL of medium containing 0.01–10 μM of PTB, 0.001–1 μM of ATRA or 9-cis RA was added. Final concentration of DMSO was adjusted to 0.1%. After a 72-h incubation at 37°C, cells were collected and stained by RPE-conjugated mouse anti-human CD11b/Mac-1 monoclonal antibody (Becton Dickinson, Cat. No. 555388) or the R-PE-conjugated mouse IgG1, κ monoclonal immunoglobulin isotype control (Becton Dickinson, Cat. No. 555749). Flow cytometry was performed by an EPICS ELITE and the results were analyzed using EPICS ELITE EXPO32 software. Differentiated cells were identified as the CD11b-positive cells.

4.5. HL-60 proliferation

HL-60 cells suspended in 100 μL of medium were seeded in a 96-well plate at 40,000 cells/well and 100 μL of medium of containing 0.003–10 μM of PTB or 0.03–30 nM of ATRA was added. Final concentration of DMSO was adjusted to 0.1%. After a 72-h incubation at 37°C, cell numbers were measured using CellTiter-Glo™ luminescent cell viability assay. Percentages of the net growth were calculated according to the following formula: 100 × (Test cell numbers – 40,000)/DMSO cell numbers – 40,000. Gl50 values were determined by non-linear curve fit analysis using GraphPad Prism.

4.6. NB4 xenograft tumor growth in vivo

The animal experimental procedures described in this study were approved by Animal Welfare Committee in Novartis Institutes for BioMedical Research Tsukuba. A 100 μL of NB4 cell suspension containing 3 × 10^6 cells was inoculated subcutaneously into the left flank of mice. Treatment was started when tumor volumes had reached approximately 70 mm^3. PTB was suspended in 0.5% CMC and administered orally once daily for 7 days. Tumor volume was calculated according to the formula: length x width^2/2.

Declarations

Author contribution statement

Chie Koshiishi, Takanori Kanazawa, Shinji Hatakeyama: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Eric Vangrevelinghe: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Toshiyuki Honda: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare the following conflict of interests: All authors are or were employees of Novartis Pharma at the time of the work was carried out.

Additional information

The datasets used in the current study are available from the corresponding author by request.

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