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TRC4, an improved triptolide derivative, specifically targets to truncated form of retinoid X receptor-alpha in cancer cells

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ABSTRACT

The nuclear retinoid X receptor-α (RXRα) plays critical roles in cell homeostasis and in many physiological processes mainly through its transcriptional function. However, an N-terminal truncated form of RXRα, tRXRα, was frequently described in various cancer cells and tumor tissues, thus representing a new promising drug target. We recently demonstrated that triptolide (TR01) could target to the oncogenic activity of tRXRα. To improve its tumor selectivity, we developed several TR01 derivatives by introducing different amine ester groups on C-14-hydroxyl site. Interestingly, C-14 modification could differently affect the expression of tRXRα without interfering the level of its full length RXRα. Among the derivatives, TRC4 could strongly reduce tRXRα expression, while TRC5-7 increased it. The capability of inhibiting tRXRα expression was shown to be closely associated with its inactivation of AKT and induction of apoptosis in various cancer cells. Conversely, treatment of cancer cells with the tRXRα-stabilizing compounds TRC5-7 resulted in enhanced AKT activity and apoptosis-resistance. However, although TR01 could strongly reduce tRXRα expression and AKT activity, it also strongly inhibited the expression and transcriptional activity of RXRα in normal cells. Importantly, the tRXRα-selective TRC4 that did not significantly inhibit RXRα transcriptional function retained the most potency of the anticancer effect of TR01 and had no significant effect on the viability of normal cells. In conclusion, our results demonstrated that tRXRα-selective TRC4 will have potential clinical application in terms of drug target and side effects. Our findings will offer new strategies to develop improved triptolide analogs for cancer therapy.

1. Introduction

Nuclear receptors (NRs), the largest family of eukaryotic transcription factors, mediate a wide range of cellular processes in response to steroid hormones, vitamins, retinoids and fatty acid derivatives [1,2]. The nuclear retinoid X receptors (RXRs) [3] are essential for retinoid signalling through forming homodimers or heterodimers with retinoic acid receptors (RARs) [4]. RXR can also heterodimerize with more than 1/3 other NR members, thus playing a unique role for cross-talk between retinoid signalling [5] and various endocrine and non-endocrine signalling [6,7]. The potential of mediating multiple functions by RXR is also revealed by the diversity of its ligands [8]. 9-*cis*-RA was originally identified as a cognate agonist of RXR [9]. Subsequently, several dietary fatty acids were found to bind RXR and act as natural RXR ligands. These include docosahexaenoic acid (DHA) [10], oleic acid [11], phytanic acid [12], and NSAID R-etodolac [13]. In addition, a number of synthetic retinoids, such as Targretin®/Bexarotene, can act as RXR ligands [14]. Some of them have been successfully developed in clinic to treat cancers and other human diseases [15,16].

Given its versatile roles in cell processes and homeostasis, altered RXR signallings are implicated in various human diseases. RXR has three subtypes (α, β, γ) but with no subtype-selective ligands identified. RXRα has been widely explored in the pathogenesis of cancer. Conditional disruption of RXRα in prostate and skin leads to preneoplastic lesions and tumors in both organs [17,18]. RXRα expression is diminished and associated with the

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development of thyroid carcinoma [19]. Impaired RXRα function by phosphorylation plays a role in the development of human hepatocellular carcinoma [20] and colon cancer [21]. An N-terminally truncated RXRα, namely tRXRα, was recently described to be frequently detected in many malignant tumors including liver and breast cancer [22]. Since tRXRα is translocated to cytoplasm, it loses transcriptional function but gains oncogenic activity due to its interaction with p85α and activation of AKT [22]. It is anticipated that specifically targeting to tRXRα will be of therapeutic significance. Thus, CF31, a new natural xanthone isolated from *Cratoxylum Formosum Ssp. Pruniflorum* [23], and K-80003 [24], a synthetic analog of NSAID sulindac, were shown to inhibit the oncogenic activity of tRXRα. Therefore, the altered RXRα expression and function represent important targets for pharmacologic interventions and therapeutic applications.

Natural products from traditional medicinal and dietary plants are rich sources for new drug discovery [25]. The clinical anticancer drugs of paclitaxel [26], etoposide [27], camptothecin [28] and vincristine [29] are the excellent examples. Triptolide, a key component of traditional Chinese medicine *Tripterygiumwilfordii* Hook. F., is biologically active against inflammation, autoimmune diseases and various human malignant tumors [30–32]. However, its severe toxicity and water-insolubility have greatly limited its clinical use [33]. To overcome this issue, many triptolide derivatives have been developed. It is no doubt that identification of molecular target for triptolide will be helpful for directing its modification and improving its application. Since the C-14-hydroxyl group of triptolide structure determines its biological outcome and solubility, many derivatives have been designed to modify this group [34–36]. Thus, substituting the C-14-hydroxylgroup with fluoride was demonstrated to be able to keep the antitumor activity while lowering the side effects [36,37]. We previously synthesized a series of novel water-soluble triptolide derivatives by replacing the C-14-hydroxyl group with amine esters [34]. As RXRα/tRXRα were demonstrated to be potential target for triptolide's action [38], we thus attempted to identify tumor-selective triptolide derivatives that may specifically target tRXRα rather the full-length RXRα. Interestingly, the apoptotic induction of these compounds was closely associated with their capability to inhibit tRXRα expression, while inactivation of RXRα transcriptional activity has a toxicity to normal cells. Fortunately, we successfully identified TRC4 as a water-soluble and tRXRα-selective triptolide derivative, which retains potent anticancer activity but does not significantly affect the growth of normal cells. Therefore, our findings provide a new direction for developing improved triptolide analogs for cancer therapy.

2. Materials and methods

2.1. Reagents

The reagent used in this study included: lipofectamin 2000 from Invitrogen (Carlsbad, CA); enhanced chemiluminescence (ECL), anti-rabbit and anti-mouse secondary antibodies conjugated to horseradish peroxidase from Thermo (Rockford, IL); anti-pAKT (ser473) (cst-4060), and anti-β-actin(A5441), anti-PARP (#9542) and anti-PCNA (#2586) from Cell Signaling Technology (CST); anti-Cyclin D1 (sc-20044), anti-RXRα (ΔN197, sc-774), anti-Bax (6A7, sc-23959), anti-Akt1/2/3 (sc-8312), anti-rabbit fluorescein isothiocyanate (FITC) and anti-mouse Cy3 from Santa Cruz Biotechnology (Santa Cruz, CA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4,6-Diamidino-2-phenylindole (DAPI) from Sigma; cocktail of proteinase inhibitors (11-873-580-001) from Roche; Dual-Luciferase Assay System Kit from Promega.

2.2. Chemistry

We introduced amine ester groups into the C-14 hydroxyl site of parental TR01 structure with the products named as TRC1-7 following our previous work [34]. The C-14 hydroxyl group was replaced with different sizes of alicyclic amine esters for TRC1-2 and TRC6, aliphatic chain amine esters for TRC3-4 and TRC7, and aralkyl amine ester for TRC5 (Fig. 1). The compounds were fully characterized by HRMS and NMR (^{1}H and ^{13}C). The purity of the compounds was more than 99% by mass spectrometry and ^{1}H NMR.

2.3. Cell culture and transfection

The cell lines used in this study included: HepG2 liver cancer cells (ATCC HB-8065), MCF-7 breast cancer cells (ATCC HTB-22), QSG-7701 liver cells, SGC7901 gastric cancer cells and GES-1 gastric cells (Institute of Biochemistry and Cell Biology, SIBS, CAS), and HEK293 cells (ATCC CRL-11268). All the cell lines were authenticated by the vendors. The cell lines were obtained between 2010 and 2013. The newly received cells were expanded and aliquots of less than 10 passages were stored in liquid nitrogen. All cell lines were kept at low passage, returning to original frozen stocks every 6 months. During the course of this study, cells were thawed and passaged within 2 months in each experiment. The cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum. Subconfluent cells with exponential growth were used throughout the experiments. Transfections were carried out by using Lipofectamine 2000 according to the instructions of the manufacturer.

2.4. Western blotting

The cell lysates were electrophoresed on 8% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked in 5% milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) for 30 min and incubated with primary antibody in 5% BSA in TBST for overnight at 4 °C. The membranes were washed three times with TBST, and incubated for 1 h at room temperature in TBST containing horseradish peroxidase-linked anti-mouse or rabbit IgG. After washing with TBST for three times, the immune-reactive bands were detected by ECL.

2.5. Immunofluorescence microscopy

HepG2 cells were seeded on cover slips in 24-well plates overnight and transfected with mock or CA-AKT vectors for 24 h. The cells were treated with triptolide or TRC4 at 50 nM in serum free condition for 12 h. The cells were fixed in 4% paraformaldehyde in PBS for 10 min, washed twice with PBS, and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. The fixed cells were pre-incubated for 30 min in PBS containing 5% bovine serum albumin. The slides were stained with anti-Bax 6A7 and anti-AKT antibodies, both at 1:50 dilution, for 1 h at room temperature, followed by incubation with secondary antibody conjugated with FITC and Cy3 (1:200 dilution). The cells were co-stained with DAPI (0.1 μg/ml) to visualize nuclei. Fluorescence images were collected and analyzed using a Zeiss LSM 510 laser-scanning confocal microscope.

2.6. Reporter assays

HEK293T cells were seeded at 3×10^4 cells per well in 48-well plates and transfected with either pGL3-promoter-RXRE luciferase reporter vector (50 ng/well) together with Myc-RXRα (30 ng/well). For pBIND reporter assays, HEK293T cells were transfected with