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Retinoid *N*-(1*H*-benzo[d]imidazol-2-yl)-5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide induces p21-dependent senescence in breast cancer cells

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ABSTRACT

Retinoids have been implicated as pharmacological agents for the prevention and treatment of various types of cancers, including breast cancers. We analyzed 27 newly synthesized retinoids for their bioactivity on breast, liver, and colon cancer cells. Majority of the retinoids demonstrated selective bioactivity on breast cancer cells. Retinoid **17** had a significant inhibitory activity (IC_{50} 3.5 μM) only on breast cancer cells while no growth inhibition observed with liver and colon cancer cells. The breast cancer selective growth inhibitory action by retinoid **17** was defined as p21-dependent cell death, reminiscent of senescence, which is an indicator of targeted receptor mediated bioactivity. A comparative analysis of retinoid receptor gene expression levels in different breast cancer cells and IC_{50} values of **17** indicated the involvement of Retinoid X receptors in the cytotoxic bioactivity of retinoid **17** in the senescence associated cell death. Furthermore, siRNA knockdown studies with RXRγ induced decrease in cell proliferation. Therefore, we suggest that retinoid derivatives that target RXRγ, can be considered for breast cancer therapies.

1. Introduction

Vitamin A and its synthetic and naturally occurring derivatives, known as retinoids, are essential in embryonic development and in the maintenance of physiological processes involving vision, metabolism, cellular homeostasis and the growth and differentiation of many tissues [1–3]. Retinoid signaling also plays an important role in carcinogenesis in transformed cells [4]. Animal experiments, cellular models and clinical trials have supported the idea of using retinoids as chemopreventive and chemotherapeutic agents [5]. All-trans-retinoic acid (ATRA), as the most-active metabolite, has been reported to affect diverse biological activities, including breast cancer [6]. ATRA inhibits cell growth in cancer cells by blocking the G1 phase of the cell cycle [7,8]. G1 arrest is induced by the induction of cyclin-dependent kinase (CDK) inhibitors such as p21 and p16 in the presence of ATRA [9,10]. CDK inhibition by p21 causes dephosphorylation of the retinoblastoma protein, Rb, leading to the inhibition of the E2F transcription factor. Consequently, target genes involved in cell cycle progression and cell proliferation are down-regulated [11]. It has been recently shown that ATRA induces cellular senescence in HepG2 cells through p21 and p16, and in MCF7 cells only with p21 activation [12].

Breast cancer is the second most common cancer worldwide and it affects about one in 10 women [13]. As a consequence of the aging of world populations, this disease is a major public health problem. Previously, breast cancer was considered a disease of women in developed countries only, but incidence and mortality rates have been increasing in less-developed countries in recent years [13]. In spite of the many developments in early diagnostic and therapeutic strategies, breast cancer is still a major obstacle. It is a heterogeneous disease and is classified into luminal,

Abbreviations: ATRA, all-trans-retinoic acid; CDK, cyclin-dependent kinase; ER, estrogen receptor; RA, retinoic acid; OIS, oncogene-induced senescence; PICS, PTEN-loss induced cellular senescence; SRB, Sulforhodamine B; SABG, senescence-associated β-galactosidase; siRNA, small interfering RNA; IC_{50}, 50% growth-inhibitory concentration; IC_{100}, 100% growth-inhibitory concentration; RAR, retinoic acid receptor; RXR, Retinoid X receptor; SERMs, selective ER modulators; TNBC, triple-negative breast cancer; SCP, senescent cell progenitor.

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basal-like, normal-like and ERBB2-positive subtypes. Estrogen-receptor (ER)–positive breast cancer cells produce senescent progeny, and this ability is correlated with ER loss and p21 accumulation [14]. Several studies have shown that retinoic acid (RA) inhibits cell growth, especially in estrogen-receptor–positive breast cancer cells, by either apoptosis or cell cycle arrest [15,16]. Retinoids have been explored as therapeutic and preventive agents in different cancer types [17–19]. In breast cancer, retinoids, especially fenretinide (4-HPR), have been investigated as preventive agents in various clinical trials [20].

Most anti-cancer agents inhibit growth by interfering with signaling pathways in cancer cells, ultimately leading to apoptosis. Recent studies indicate that drug-dependent senescence is a promising mechanism that may advance cancer therapy [21,22]. During cellular senescence, cells grow old and die due to aging. Involving novel chemotherapeutic candidates in reprogramming cell senescence is an important approach in the realm of cancer treatment. Normally replicative senescence observed due to telomere shortening during replication whereas the molecular analysis of senescence in cancer cells demonstrated oncogene-induced senescence (OIS) and PTEN-loss induced cellular senescence (PICS) mechanisms [23–25]. Based on these findings and due to the prolonged activity of retinoids in the cell, we submit that these compounds be further exploited as senescence-associated anti-proliferative agents in chemotherapeutic regimes.

In this study, we tested previously synthesized retinoid derivatives [26] for their cytotoxicity in a series of breast cancer cell lines. We then further studied compound **17**, which showed the most anti-proliferative activity, to identify its mechanism of action at the molecular level.

2. Experimental

2.1. Cell culture

The breast cancer cell lines (Cama-1, T47D, MCF7, BT-474, MDA-MB-453, BT-20, SK-BR-3, MDA-MB-361, MDA-MB-157, MDA-MB-231 and MCF-12A) were obtained from ATCC. All breast, Huh7 liver and HCT116 cells were authenticated by STR analysis regularly. T47D, BT-474, MCF-7, BT-20, MDA-MB-453, MDA-MB-231 and Huh7 were grown in Dulbecco's modified Eagle's medium (DMEM). Cama-1 and MDA-MB-157 were cultivated in DMEM supplemented with 1% sodium pyruvate. SK-BR-3 was cultivated in RPMI (glucose rich; 4.5 g/L) medium (Sigma). Unless indicated all media had phenol red and supplemented with 10% FCS and 50 mg/ml penicillin–streptomycin for both retinoid treated and control experiments. Compound **17** was further tested in phenol red free medium in order to validate its cytotoxic activity in T47D cells. This study does not involve animals or human volunteers therefore ethics approval is not required.

2.2. Preparation of the compounds

Retinoids were kept in powder form at dark 4 °C and they were dissolved in Dimethyl sulfoxide (DMSO) with a concentration of 20 mM the stock solution of the compounds were prepared and kept in −20 °C during the experiments. For SRB assay concentration curve from 40 μM, 20 μM, 10 μM, 5 μM to 2.5 μM were used. For other experiments the concentrations were used as indicated in the figure legends. The retionids were prepared from the stock solutions prior to the experiments. Because retionids are light sensitive, compounds were always kept in dark and experiments were done under dim light.

2.3. Sulforhodamine B (SRB) cytotoxicity assay

Retinoids were tested with an National Cancer Institute (NCI) anticancer drug screening method for their growth-inhibitory activity [27]. The cells (10,000 cells/well) were seeded into 96-well plates in 200 μl of medium 24 h prior to treatment with retinoids. After 72 h of treatment with retinoids, the cells were fixed by 60 μl of cold TCA (10% (w/v)) for 60 min at 4 °C. Then 100 μl 0.4% SRB solution was applied and the cells were incubated for 10 min at room temperature. Unbound dye was washed five times with 1% acetic acid and air dried. An SRB dye solubilized by 10 mM Tris-Base solution and absorbance were acquired at 515 nm. Absorbance values of DMSO only treated wells, which were controls, were used for normalization. 50% growth-inhibitory concentration (IC_{50}) values were calculated as described in [28].

2.4. Senescence-associated β-galactosidase (SABG) assay

T47D cells were seeded onto coverslips in 12-well plates as 7500 cells/well. After 24 h compound **17** was added to the wells as at IC50 (3.7 μM) and IC100 (7.4 μM) levels. Control wells were treated with only DMEM or same drug level of DMSO. Every 48 h cell culture medium and the drug was replenished. Experiments were stopped at 2th, 4th and 6th days of treatment and then SABG assay was performed. Experiments were generated as a triplicate for each condition [29]. Cells were counterstained with nuclear fast red following SABG staining. SABG positive and negative cells from each condition were counted under the light microscope from randomly selected areas and percentages were calculated for SABG positive and negative cells.

2.5. Western blot analyzes

Upon treatment with compound **17**, cell pellets were incubated in an NP-40 lysis buffer (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 0.1% Nonidet P-40) and a protease-inhibitor cocktail (Roche) for 30 min at 4 °C. Bradford assay was performed to quantify the protein concentration of the cell lysates. 30 μg of protein was denatured and resolved by SDS–PAGE using 10% gel. Then the proteins were transferred to the nitrocellulose membranes. Membranes were treated for 1 h with blocking solution (TRIS-buffered saline containing 0.1% Tween-20 and 5% non-fat milk powder (TBS-T)) and probed with a primary antibody for 1 h. Next, membranes were washed three times with TBS-T and incubated with an HRP-conjugated secondary antibody for 1 h. Then immune complexes were detected by an ECL-plus (Amersham) kit. Calnexin and Actin were used for equal loading control. The following antibodies were used in this study: anti-p21Cip1 (OP64; Calbiochem), Rb (BD Bioscience, 554136), phospho-Rb (Ser 807/811) (Cell Signaling, 9308S), Calnexin (Sigma, C4731) and Actin (Santa Cruz, sc1616).

2.6. RNA extraction, cDNA synthesis and semiquantitative RT-PCR

Total RNA was extracted from cultured cells with a Nucleo Spin RNA II Kit (MN Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. Two micrograms of total RNA were reverse transcribed into cDNA in a total volume of 20 μl using a Revert Aid First Strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania). The PCR reactions were carried out with 1 μl of cDNA, using the appropriate number of cycles and annealing temperature (Tm). Annealing temperatures (Tm) and cycle numbers were optimized for each transcript. The PCR conditions were: RAR-α; Tm: 55 °C, 30 cycles, RAR-β; Tm: 60 °C, 30 cycles, RAR-γ; Tm: 60 °C, 32 cycles, RXRα and RXRβ; Tm: 58 °C, 30 cycles, RXRγ; Tm: 62 °C, 35 cycles. The primer sequences were: RAR-α F-5′GAGCCGGTCCTTTGGT CAA3′, R-5′CTGCGAGCATCACAGGACAT3′, RAR-β F-5′ATTCCAGTGC