Retinoid N-(1H-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

Mine Mumcuoglu [a,b], A. Selen Gurkan-Alp [c], Erdem Buyukbingol [c], Rengul Cetin-Atalay [a,d,*]

[a] LOSEV the Foundation for Children with Leukemia, Cancer Genetics Research Laboratory, Ankara, Turkey
[b] Department of Molecular Biology and Genetics, Bilkent University, Bilkent, 06800 Ankara, Turkey
[c] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ankara University, Turkey
[d] Graduate School of Informatics, Cancer Systems Biology Laboratory, METU, 06800 Ankara, Turkey

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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,

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 antiproliferative 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

TGACCATCGAGTCC-3′, R-5′CCTGTTTCTGTGTCATCCATTTCC3′, RAR-γ F-5′TACCACTATGGGGTCAGC3′, R-5′CCGGTCATTTCGCACAGCT3′ RXRα, F-5′TTCGCTAAGCTCTTGCTC3′, R-5′ATAAGGAAGGTGTCAAT GGG3′ RXRβ, F-5′GAAGCTCAGGCAAACACTAC3′, R-5′TGCAGTCTTT GTTGTCCC3′ RXRγ, F-5′GCAGTTCAGAGGACATCAAGCC3′ and R-5′GCCTCACTCTCAGCTCGCTCTC-3′. PCR products were analyzed on a 2% agarose gel and visualized with ethidium bromide under UV transillumination. The mRNA bands were quantified by the Image J program (http://imagej.nih.gov).

2.7. siRNA transfection

siRXRα (Dharmacon) and siRXRγ (Invitrogen) were transfected into MCF7 cells by using the "reverse transfection" method according to the manufacturer's guidelines. siRNA silencing was then confirmed by RT-PCR semiquantitative RT-PCR analysis.

2.8. Statistical analysis

Statistical analysis was carried out with StatPlus:mac software (AnalystSoft). Statistical differences between two groups were determined using the student's t test, and between all groups were determined with an analysis of variance (ANOVA) test with a Bonferroni adjustment. p values of <0.05 or $p < 0.01$ were accepted as statistically significant.

2.9. Static docking

The coordinate files of the human retinoic acid receptors RXRγ and RXRα ligand-binding domains (pdb ID: 2GL8 and 1FBY) were prepared for docking with the UCSF Chimera tool, a visualization system for exploratory research and analysis [30]. The Chimera Dock Prep tool was used to delete water molecules, to add hydrogen and to write the file in Mol2 format from the homotetramer RXRγ structure. A 3D structure of compound 17 was prepared in Mol2 format with Marvin Sketch (http://www.chemaxon.com) and the retinoic acid structure was acquired from Protein Data Bank (pdb ligand ID: REA). Docking between RXRs and compound 17 was performed on the SwissDock server based on EADock DSS [31,32]. Binding modes were analyzed using the Chimera tool; the docked 2GL8 for RXRγ and 1FBY for RXRα are shown in Fig. 5.

3. Results

3.1. Cytotoxic activity of retinoids in cancer cells

Twenty-seven newly synthesized retinoid derivatives [26], N-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalene-2-yl)-carboxamides (6-15) and 5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamides (16-32) were screened for their cytotoxic activity in cancer cells by the SRB assay. T47D breast, Huh7 liver and HCT116 colon cancer cells were treated with the compounds and with ATRA, and their IC$_{50}$ values were determined (Supplementary Table 1 and Supplementary document 1).

The cytotoxicity induced by the compounds was prominent in T47D cells. The compounds with the lowest IC$_{50}$ concentrations (6, 8, 10, 11, 17, 18, 24, 25, 26, and 27), were selected to be further analyzed for their anti-proliferative activity on a larger panel of breast cancer cells and on an MCF-12A immortalized normal breast epithelial cell line (Table 1 and Supplementary document 2). Compounds 6, 8, 10 and 11 have been previously synthesized as derivatives of N-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-carboxamides, compounds 17, 18, 24, 25, 26 and 27 have been synthesized as derivatives of 5,5,8,8-tetramethyl-

5,6,7,8-tetrahydronaphthalene-2-carboxamides [26]. Camptothecin, which is a potent cytotoxic agent, was included as an experimental positive control (Table 1).

The selected compounds had heterogeneous cytotoxic actions on breast cancer cell lines. Compounds 17 and 24 had the lowest IC$_{50}$ values in certain cell lines (Table 1). The cytotoxicity of compound 24 was more specific on BT-20 cell lines, while compound 17 exhibited broader cytotoxic activity on breast cancer cells. The 50% growth-inhibitory concentration of compound 17 on MCF-12A, which is the immortalized normal breast epithelial cell line, was fivefold higher than that of T47D. Additionally, in a previous study, compound 17 was found to be non-toxic normal human macrophage cells [26]. Therefore, compounds 17 and 24 are considered good cytotoxic agent candidates for the treatment and prevention of breast cancer. Compound 17 had no cytotoxic action on the Huh7 liver and HCT116 colon cells (Supplementary Table 1). Based on these observations, we focused our studies on the cytotoxicity induced by 17, which showed the most breast-cancer-cell specificity within the 27 tested retinoid derivatives. We also confirmed the cytotoxic activity of retinoid 17 in phenol red free medium in comparison with DMSO controls on T47D cells. SRB-cytotoxicity assay was performed in triplicate and IC$_{50}$ value were identified as 9.8 μM ($R^2 = 0.9$). When the IC$_{50}$ value (3.5 μM) in the presence of phenol red which induces mild cell proliferation, compared to this value (9.8 μM), we observe that 17 may be more active against fast proliferating cells.

3.2. Identifying the cell death type induced by 17

To understand the molecular mechanism behind the cytotoxic effect of compound 17 (Fig. 1A), we first determined whether this action occurs by apoptosis. To test apoptosis induction, we performed in situ Hoechst-33258 staining, cytochrome C release and PARP protein cleavage analysis (Supplementary Fig. 2) after treating T47D cells with compound 17. None of these apoptosis markers gave positive results.

Also observed during compound 17 treatments of breast cancer cells was a late cytotoxic response, which is indicative of long-term cell death induction. An anti-proliferative effect was seen after about six days of retinoid treatment. This late response led us to consider senescence-associated cell death, which is another important growth-inhibitory mechanism observed in cancer cells [33]. Therefore, we investigated whether senescence induction occurs upon treating T47D cells with compound 17. SABG staining was performed after two-, four- and six-days of treatment with 17 on T47D cells. Compound 17 was applied to the cells at IC$_{50}$ (3.7 μM) and IC$_{100}$ (7.4 μM) concentrations. SABG-positive and negative cells were counted from randomly selected areas under the light microscope and the percentages of senescent cells were calculated. The IC$_{100}$ concentration of 17 caused statistically significant senescence associated cell death compared to DMSO controls (Fig. 1B). Representative pictures of SABG staining are presented in Fig. 1C. Blue cells demonstrate SABG positivity as a result of senescence-associated cell death.

3.3. Molecular analysis of senescence upon 17 treatment

CDK inhibitor p21waf1/Cip1/Sid1 is an important mediator of p53-dependent cell cycle arrest upon DNA damage [10,34]. p21 is also one of the key regulators of senescence, and ATRA treatment causes up-regulation of p21 in different cell lines [35]. These findings motivated us to determine the involvement of the p21-Rb pathway in compound 17's mechanism of action. For this purpose we analyzed p21 protein levels in T47D cells treated with IC$_{50}$ and IC$_{100}$ concentrations of 17 for two, four and six days. These treatments induced a time-dependent p21 protein increase for

Table 1
Cytotoxic activities (IC$_{50}$ in μM) of the retinoids on breast cancer cell lines.[a]

Cell lines	Compounds										
	6	8	10	11	17	18	24	25	26	27	CPT[b]
CAMA-1	38.11	24.94	4.82	23.44	12.16	8.73	7.28	9.6	7.47	9.97	0.07
T47D	17.3	12.08	14.93	16.43	**3.71**	6.03	**4.08**	10.31	10.5	8.67	<0.01
MCF7	8.07	9.66	9.02	11.1	6.93	12.98	10.5	22.09	11.57	8.8	<0.01
BT474	16.52	7.81	26.35	22.01	12.91	8.37	NI[c]	21.38	NI	14.54	12.75
MDA-MB-453	11.97	5.9	13.6	23.33	6.19	16.92	17.48	20.57	9.03	12.33	<0.01
BT20	11.33	10.05	11.62	11.61	**3.85**	11.68	**2.55**	9.46	14.52	11.27	0.07
SK-BR-3	9.77	7.46	10.1	7.72	11.23	8.38	NI	18.68	NI	9.68	<0.01
MDA-MB-361	11.18	8.58	9.76	15.07	11.45	11.06	NI	NI	NI	12.15	0.14
MDA-MB-157	18.81	19.49	12.85	16.94	9.77	22.62	NI	NI	14.21	7.07	0.02
MDA-MB-231	42.85	18.93	15.22	15.7	32.6	16.57	24.99	NI	13.32	12.98	<0.01
MCF-12A	17.93	NI	10.86	11.4	15.91	NI	NI	NI	34.62	NI	<0.01

Significant IC$_{50}$ are indicated in bold.
[a] Cytotoxicities of all 27 retinoids are given in Supplementary Table 1.
[b] CPT: Camptothecin is used as an experimental positive control.
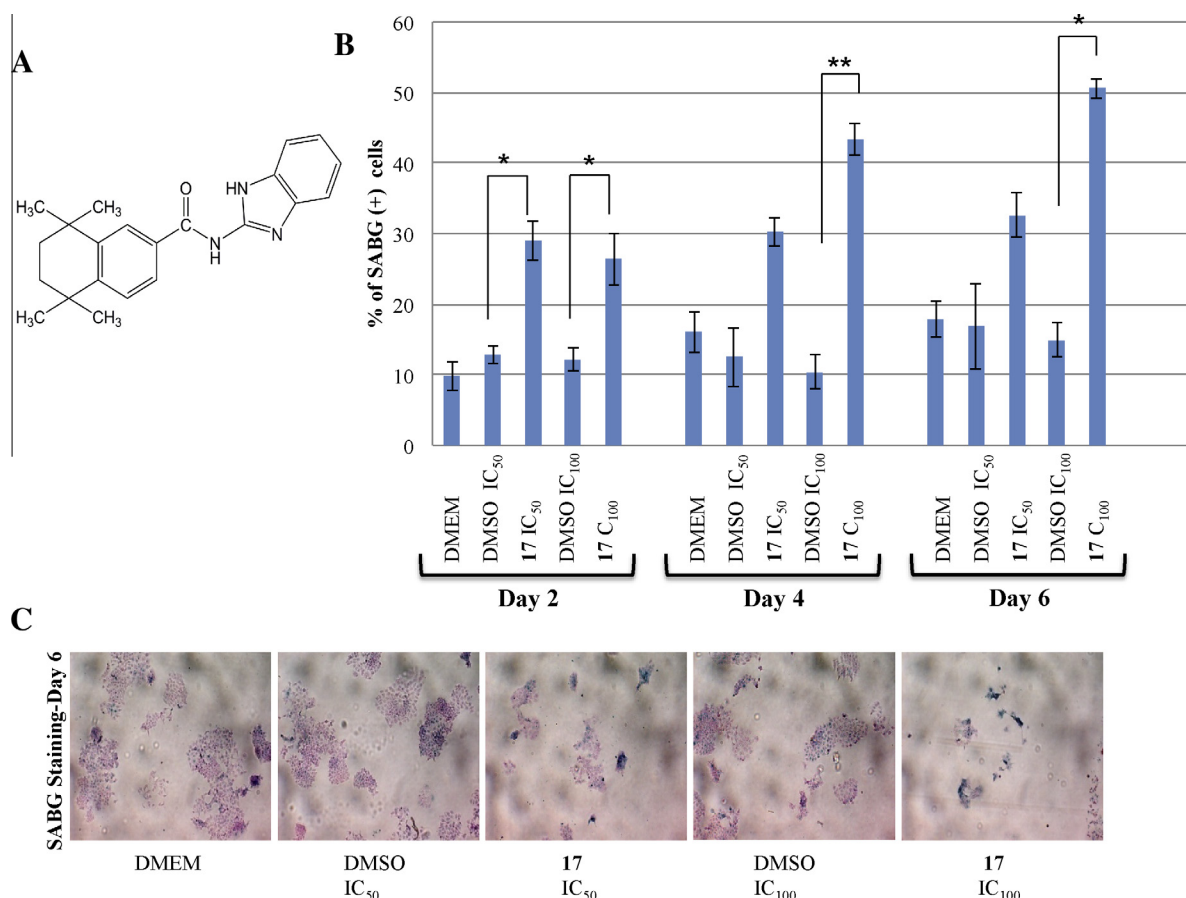[c] NI: no inhibition.

Fig. 1. Compound **17** leads to senescence in T47D breast cancer cells. (A) Chemical structure of compound **17**. (B) T47D cells were treated with IC$_{50}$ and IC$_{100}$ concentrations of **17** for 2, 4 and 6 days and subjected to an SABG assay. SABG-positive cells were counted and the percent of senescent cells was presented. (C) SABG-stained in T47D cells upon 6 days of treatment with IC$_{50}$ (3.7 μM) and IC$_{100}$ (7.4 μM) concentrations of **17**. *p*-Values were calculated by paired two-tailed *t* tests. *$p < 0.01$, **$p < 0.005$.

both concentrations; in comparison, DMSO-treated control cells (Fig. 2).

3.4. Correlation between retinoic acid receptors and the cytotoxicity of 17

The variation observed in the IC$_{50}$ values motivated us to further analyze the molecular mechanisms in senescence and its possible relation to RARs. Differential expressions of RAR levels in breast cancer cells might be the reason for these cells' diverse

IC$_{50}$ values obtained with the retinoid derivatives (Table 1 and Fig. 3A). Retinoic acid and its synthetic derivatives (retinoids) mediate their effect through RARs and RXRs. Therefore, knowledge of RAR expression in our breast cancer cell line panel was essential in identifying the subtype-specific cytotoxicity induced by **17**. For this purpose, the cells were characterized for their RAR and RXR status by the semi-quantitative RT-PCR method (Fig. 3 and Supplementary Fig. 3). We did not observe an association between IC$_{50}$ values and RAR expression levels (Fig. 3B). Although RXRα and RXRβ were expressed equally in all cell lines tested, RXRγ
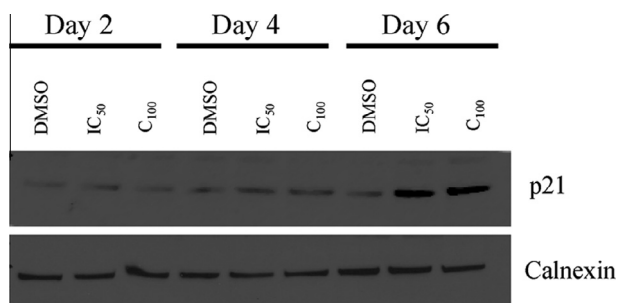
Fig. 2. Senescence-associated upregulation of p21. T47D cells were treated with compound **17** at IC_{50} (3.7 μM) and IC_{100} (7.4 μM) concentrations and in DMSO controls. p21 protein levels were then analyzed on days 2, 4 and 6 with Western blot. During treatment, **17** was renewed every 48 h. Calnexin was used as an equal loading control.

expression correlated with the cytotoxicity induced by **17** (Fig. 3 and Table 1). Indeed, this correlation could be the result of the RXRγ expression and senescent progenitor subtype described previously [22]. Therefore, we knocked down RXRα or RXRγ in the MCF7 cell line, and then treated the cells with **17**. We specifically chose MCF7 cells, which were reported to be senescent progenitors, to demonstrate our hypothesis in a luminal type of breast cancer cell other than T47D.

3.5. Response of RXRα and RXRγ knocked-down MCF7 cells to 17

MCF7 cells were transiently transfected with siRXRα, siRXRγ and scrambled siRNA as a control. The transfected cells were then treated with compound **17** and subjected to an SRB cytotoxicity assay (Fig. 4A). The efficiency of the knockdown was evaluated by RT-PCR in parallel with the SRB assay (Fig. 4B and C). RT-PCR results showed that the knockdown of RXRα was 70% achieved and that of RXRγ was silenced totally. Silencing of RXRα had no effect on the proliferation of MCF7 cells, whereas RXRγ induced significant cytotoxic action ($p < 0.02$). In addition, **17** was also cytotoxic when RXRα was knocked down. These results may suggest that compound **17** maintained its cytotoxic effect despite the absence of the RXRα receptor through other retinoid receptors. (Fig. 4C).

3.6. Docking of compound 17 on RXRγ

To gain more insight into the interaction between compound **17** and RXRα or RXRγ at the molecular level, we performed small-molecule docking studies with ligand-binding domains of human retinoic acid receptors RXRα (1FBY) and RXRγ (2GL8) using Swissdock (Fig. 5A*i* and B*i*) [32]. Then we docked retinoic acid (PDB id: REA) to RXRα and RXRγ and structurally aligned it to RXRα·**17** and RXRγ·**17** to demonstrate the binding site orientation of **17** with respect to REA (Fig. 5A*ii* and B*ii*). The alignment shows that RXRγ·**17** has more structural overlap than RXRα. This observation further suggests that the cytotoxic effect of **17** may be due to its interaction with RXRγ and it can be further studied by molecular dynamics studies in parallel with in vitro direct binding assays.

4. Discussion

Retinoids have been reported to be involved in chemoprevention and chemotherapeutics in cancer [1,4–6]. Therefore, we examined the cytotoxic activities of newly synthesized 28 retinoids, including ATRA, on epithelial-origin breast, liver and colon cancer cells (Supplementary Table 1). Retinoids **6**, **8**, **10**, **11**, **17**, **18**, **24**, **25**, **26** and **27** had cytotoxic activities with IC_{50} values below

10 μM on all cancer cell types. Retinoid **17** had selective cytotoxicity on T-47D breast cancer cells but not on Huh7 liver and HCT116 colon cancer cells. Regarding the importance of the hormonal component of breast cancer, we focused on the molecular cytotoxicity analysis of retinoid **17** for a larger panel of breast cancer cells from different classes. We tested the cytotoxicity of retinoid derivatives on four Luminal A (CAMA-1, T-47D, MCF-7 and MDA-MB-361), one Luminal B (BT-474), three Triple-Negative-Basal (BT-20, MDA-MB-157 and MDA-MB-231) and two HER2 (MDA-MB-453 and SK-BR-3) subtypes of breast cancer cell lines (Table 1). Luminal A is the most frequently diagnosed subtype, corresponding to 50–60% of all breast cancers. This subgroup represents estrogen receptor positivity. Generally, the luminal subgroup of breast cancer does not respond very well to traditional chemotherapy [36,37]; the major treatment for this group depends on hormone therapy, such as selective ER modulators (SERMs), like tamoxifen, and pure selective ER regulators, like fulvestrant. Another major challenge in hormone-positive breast cancer is *de novo* or acquired resistance to hormone therapy. Resistance to hormone therapies mostly occurs through the activation of signaling pathways that give input to cell cycle progression [38]. For this reason, there is a need for alternative targets and therapies for the hormone-resistant luminal group of breast cancer as well as for other hormone-negative breast cancer types. On the other hand, targeted therapies for triple-negative breast cancer (TNBC), which has a very poor prognosis and distant recurrence, are essential. Retinoids and differential activities of retinoid receptors in breast cancer cells have been previously studied [39]. The retinoid derivative AM580 is reported to be more active on RARα than the pan-RAR ligand, ATRA. Furthermore, we also do not observe significant cell growth inhibition with ATRA (IC_{50} of 28.5-Supplementary Table 1) since ATRA does not bind to RXR. Similarly, some retinoid derivatives we tested on T-47D cells were active; some were inactive, indicating the selectivity of retinoids against RARs. As shown in Table 1, further cytotoxicity analysis of the most-active retinoid derivative, compound **17**, successfully blocked the proliferation of luminal and TNBC cells at micromolar concentrations. Retinoid **17** had IC_{50} values of 3.71 μM for T-47D Luminal A and IC_{50} values of 3.88 μM for BT-20 TNBC cells.

Our data also reveal that cell death induced by retinoid derivatives is senescence in breast cancer cells. ATRA's induction of senescence in liver cancer cells has been previously described as a p21-dependent mechanism for this cell death type [12]. Pan-retinoid ATRA or other retinoid derivatives' modes of action in inducing cell death is not clearly defined at the molecular level in breast cancer cells [39]. On the otherhand, the T-47D cell line has been grouped in a senescent cell progenitor (SCP) subtype and senescence occurrence has been reported to be associated with estrogen receptor loss and p21 accumulation in this cell line by our group [14]. In correlation with these findings, we demonstrated that compound **17** led to the accumulation of p21 and induced senescence response in this cell line. In parallel, at the end of day six, Rb levels had increased and pRb levels remained unchanged during senescence (data not shown). In conjunction with our results, ATRA has been shown to induce cell-cycle arrest through p21 overexpression in human monoblastic U-937 cells and liver cancer HepG2 cells, indicating the importance of retinoids in cancer therapy [9,40]. Recently, Lim et al. showed that ATRA causes senescence in HepG2 hepatoma cells by upregulation of p16 and p21. They also observed RAR-β2 involvement in this effect [12]. Our study with the retinoid derivative **17** on 11 different breast cancer cells and a comparative analysis of RAR and RXR gene expression reveal the importance of RXRs in breast cancer cell proliferation. RXR knockdown experiments using RXRα- and RXRγ-specific siRNAs in the presence or absence of retinoid derivative **17** showed not only the cell-survival-inhibitory effects of this compound but also
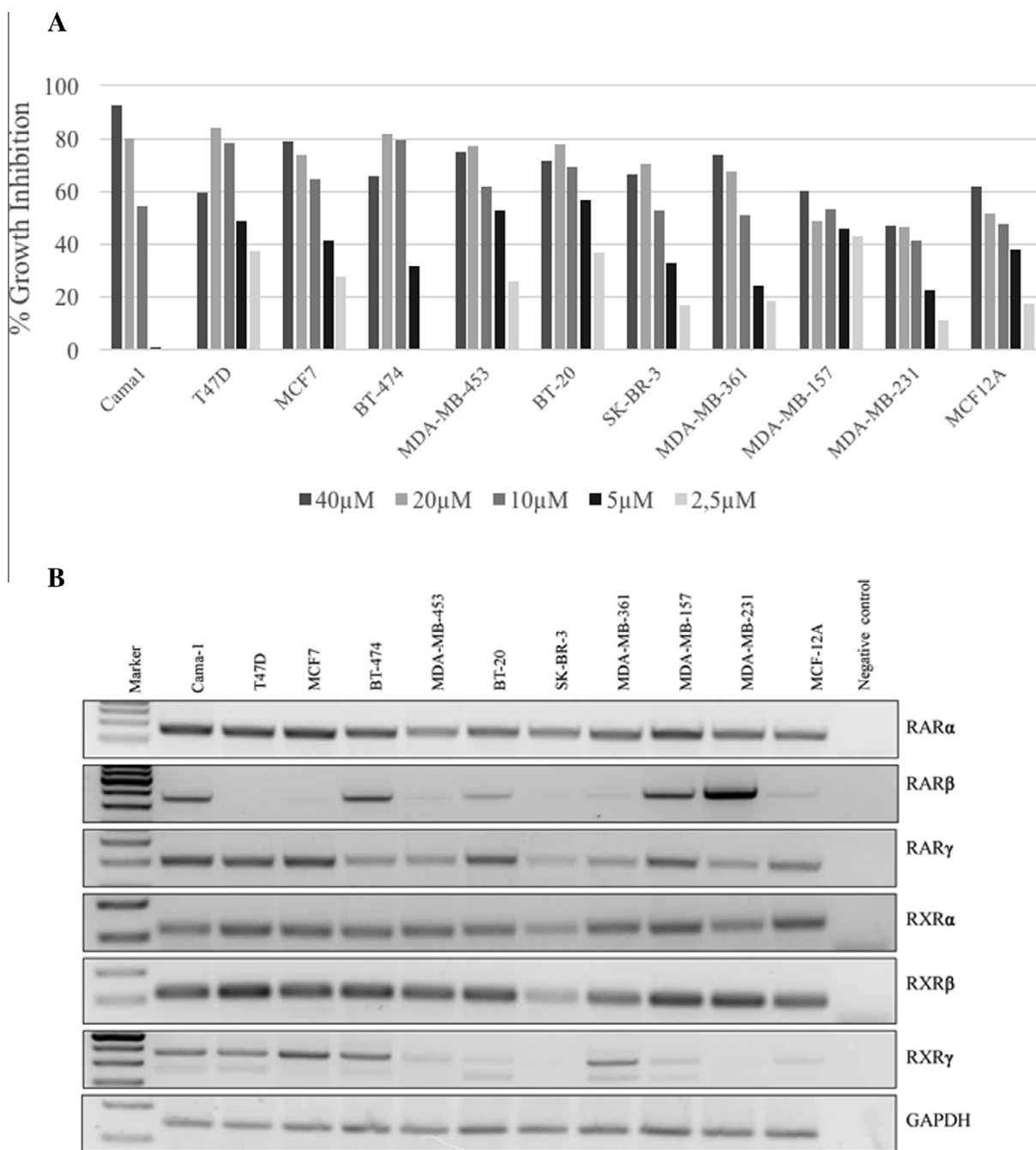
Fig. 3. Comparative analysis of the cytotoxic action of compound **17** and retinoid receptor expression. Dose response curves of **17** cytotoxicity with concentrations of **17** from 40 μM, 20 μM, 10 μM, 5 μM to 2.5 μM in DMSO and growth inhibitions were compared to DMSO controls (A). Experiments were done in triplicates and mean values were indicated in the graph. Retinoic acid receptor expression levels in breast cancer cell lines (B). Breast cancer cells were lysed, mRNA was isolated and RARα, RARβ, RARγ, RXRα, RXRβ and RXRγ levels were determined using RT-PCR. GAPDH was used for a reference gene expression.

the importance of RXRs in breast cancer cell proliferation. While siRNA-mediated RXRα knockdown did not alter cell proliferation, RXRγ knockdown had a significant anti-proliferative effect on MCF7 cells. Compound **17**, had a dramatic anti-proliferative activity on RXRα knockdown cells with normal RXRγ expression, indicating that the major target of compound **17** can be RXRγ. However we were able to knockdown RXRα only by 70% achieved which does not rule out that **17** does not target RXRα for its cytotoxic activity. Our initial docking analysis can be further investigated by molecular dynamics and direct in vitro binding assays. Similarly structurally modified two Pyrazine Arotinoid derivatives reported to be selectively differential activities on RAR and RXRs

supporting the selectivity of retinoid derivatives against retinoid receptors [41,42]. Findings of this study showed that Retinoid X receptors could be associated with the anti-proliferative effects of retinoid derivative **17**. We also demonstrated that RXRγ expression down-regulation leads to a significant decrease in cell proliferation. Therefore, we suggest that retinoid derivative **17** and other retinoids that target RXRγ, can be considered for breast cancer treatment in patients experiencing resistance to hormonal therapies. We submit that mechanisms underlying the regulation of Retinoid X receptors and small molecules' action on these proteins may merit further evaluation as a novel strategy against breast cancer.
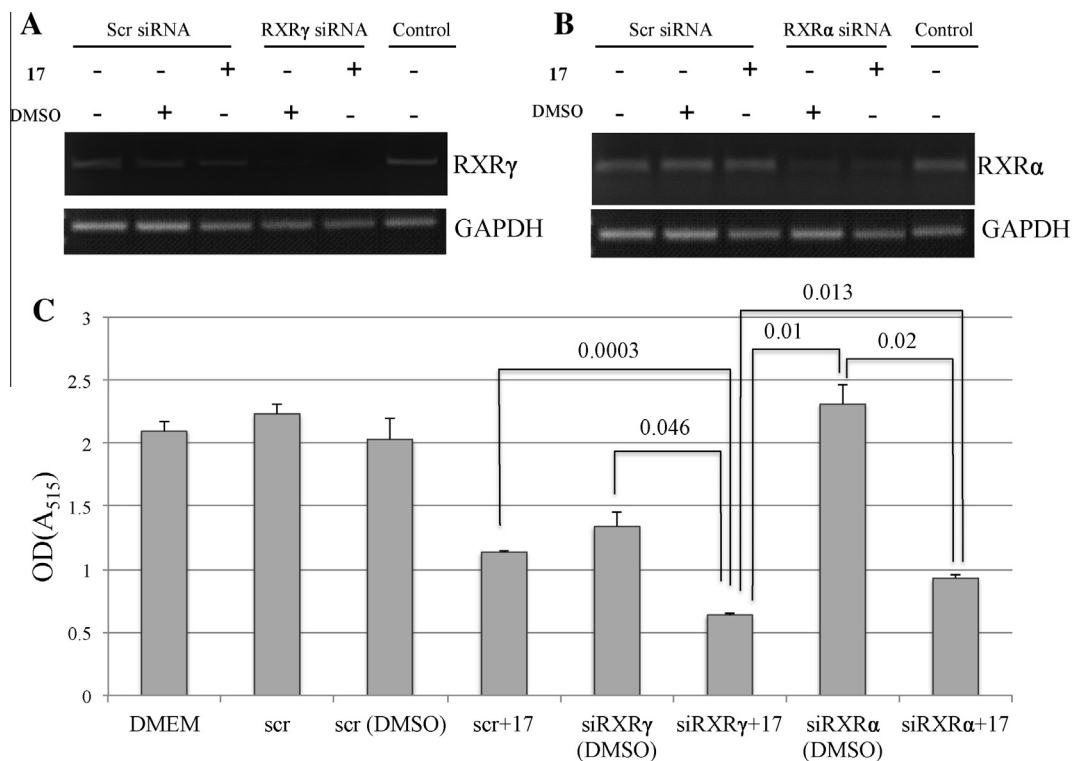
Fig. 4. Effect of compound **17** on RXRγ- or RXRα-silenced cells. MCF-7 breast cancer cells were transiently transfected with (A) siRXRα and (B) siRXRγ and scrambled siRNA, and treated with either compound **17** or DMSO. RXR knockdown was confirmed by RT-PCR analysis. GAPDH was used for a reference gene expression. (C) Cytotoxicity of **17** was analyzed by an SRB assay. Results were analyzed by an analysis of variance (ANOVA) test. Bonferroni-adjusted *p*-values were indicated between groups. Each experiment was done in triplicate.
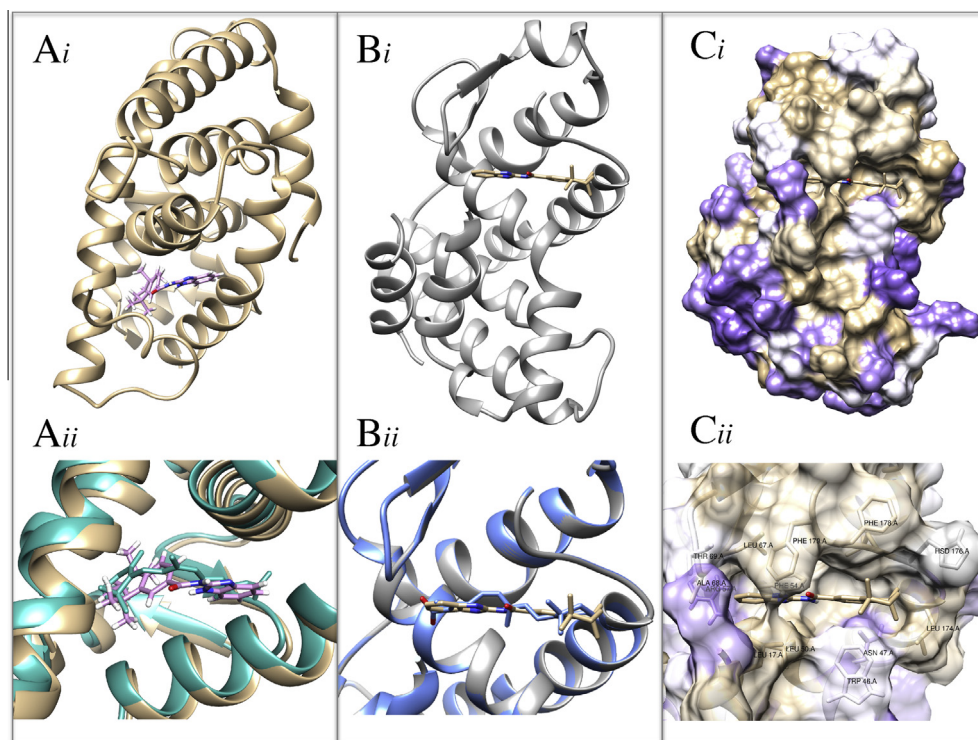

Fig. 5. Comparative representation of compound **17** docked on RXRα and RXRγ. Compound **17** was docked to the ligand-binding domains of human retinoic acid receptors: (A*i*) RXRα (PDB-ID: 1FBY) and (B*i*) RXRγ (PDB-ID: 2GL8). Retinoic acid docked RXRα and RXRγ were structurally aligned with (A*ii*) RXRα·**17** and (B*ii*) RXRγ·**17** to demonstrate the binding site orientation of **17** with respect to **REA**. RXRγ had a better structural alignment than RXRα. (C*i-ii*) Surface representation of RXRγ with **17**, where purple indicates the most-hydrophilic and tan-color indicates the most-hydrophobic regions.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2016.02.008.

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