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Src is a novel potential off-target of RXR agonists, 9-cis-UAB30 and Targretin, in human breast cancer cells

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Abstract

9-cis-UAB30 (UAB30) and Targretin are well-known RXR (retinoid X receptor) agonists. They were highly effective in decreasing the incidence of methylnitrosourea (MNU)-induced mammary cancers. However, whether the anti-mammary cancer effects of UAB30 or Targretin originate from the activation of RXR is unclear. In the present study, we hypothesized that UAB30 and Targretin not only affect RXR, but likely influence one or more off-target proteins. Virtual screening results suggest that Src is a potential target for UAB30 and Targretin that regulates ECM (extracellular matrix) molecules and cell motility and invasiveness. *In vitro* kinase assay data revealed that UAB30 or Targretin interacted with Src and attenuated its kinase activity. We found that UAB30 or Targretin substantially inhibited invasiveness and migration of MCF-7 and SK-BR-3 human breast cancer cells. We examined the effects of UAB30 and Targretin on the expression of matrix metalloproteinases (MMP)-9, which are known to play an essential role in tumor invasion. We show that activity and expression of MMP-9 were decreased by UAB30 or Targretin. Western blot data showed that UAB30 or Targretin decreased AKT and its substrate molecule p70^{s6k}, which are downstream of Src in MCF-7 and SK-BR-3 cells. Moreover, knocking down the expression of Src effectively reduced the sensitivity of SK-BR-3 cells to the inhibitory effects of UAB30 and Targretin on invasiveness. Taken together, our results demonstrate that UAB30 and Targretin each inhibit invasion and migration by targeting Src in human breast cancer cells.

Keywords

9-cis-UAB30; Targretin; Src; Breast cancer cells; Invasion and migration

Introduction

Breast cancer is the most prevalent cancer for women worldwide [1]. Mortality associated with breast cancer is not necessarily due to the original primary tumor, but usually results from metastases at distant sites. Approximately 25–40% of patients with breast cancer will

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progress to metastatic incurable disease [2]. Metastasis progresses through multiple complex steps, which include the breaking away of cells from primary tumors and their invasion through basement membranes and extracellular matrix (ECM). This is generally followed by circulation and survival in the vasculature and extravasation into remote tissues, finally resulting in the establishment of metastatic tumors [3]. The primary metastatic sites of breast cancer include lymph nodes, lungs, liver, brain, with bone being the most common site [4].

Uncontrolled degradation of the ECM and basement membrane is an essential part of the metastatic process [5] and the matrix metalloproteinase (MMP) enzyme family plays a major role in this degradation. In particular, MMP-9 is the main enzyme that degrades type IV collagen and is overexpressed in invasive tumors, suggesting that this enzyme may play a crucial role in cancer invasion through its enzymatic degradation of the ECM [6]. Thus, suppressing MMP-9 activity could be an effective therapy to inhibit invasion and metastasis of breast cancer [7].

The retinoids comprise a group of structural and functional derivatives of vitamin A. These molecules regulate many important biological processes, such as cell differentiation, growth and death; and modulation of their receptors is a promising treatment strategy against cancer, dermatological diseases, HIV infection, and type II diabetes [8]. Responsiveness to retinoids is determined by the retinoic acid receptor (RAR) and retinoid X receptor (RXR) expression. The RARs and RXRs are intracellular or nuclear receptors that function as ligand-dependent transcription factors. RAR/RXR heterodimers regulate the transcriptional activation of primary retinoic acid (RA) target genes such as *p21^{WAF1/CIP1}* by binding to DNA-response elements termed RA response elements (RAREs) [9]. Activation of target genes will trigger cell arrest and apoptosis [10,11]. Retinoids have been studied for several years as a promising class of agents in the treatment and prevention of cancer. Many studies have shown that adverse skin changes and liver toxicities are representative side effects of RA-derivatives now in clinical use and these side effects stem from activation of the RAR [12]. RAR is lost or reduced in various cancer types and therefore resistance against RAR agonists can occur [13].

To avoid the adverse effects of retinoids, RXR-selective ligands, also referred to as rexinoids, were developed. Putative synthetic RXR-selective agonists include UAB30 (Fig. 1A) and Targretin (bexarotene, Fig. 1B) [14], which have been reported to exert chemopreventive effects on breast cancer with less toxicity compared to retinoids. RXR expression is rarely lost in human tumors. Therefore, rexinoids appear to be safe and can overcome resistance [13]. UAB30 and Targretin have been shown to prevent both estrogen receptor positive and negative breast cancer cell growth in various rodent models including transgenic lines expressing C3(1)-SV40 T-antigen (Tag), overexpressing MMTV-ErbB2 (HER-2) and p53-null mice and rats [15]. Targretin has been tested in numerous clinical trials, and has already been approved by the U.S. Food and Drug Administration [13]. However, these observations cannot explain all effects of these drugs and these observations suggest that selective RXR agonists act by mechanisms other than targeting RXR. Therefore, we were interested in finding off-target molecules of these drugs. In this study, we demonstrated that the inhibitory effects of UAB30 and Targretin against invasion and

migration occurred through the attenuation of Src in ER positive (MCF-7) and HER-2 overexpressing (SK-BR-3) breast cancer cells [16].

Materials and Methods

Reagents

UAB30 and Targretin were received from Dr. Clinton Grubbs (University of Alabama at Birmingham). Dulbecco's modified Eagle's medium and other supplements were from Life Technologies, Inc. (Carlsbad, CA). The human recombinant Src, p38 and PKC δ were purchased from Millipore Corp (Billerica, MA). The antibodies against phosphorylated AKT (Ser473), total AKT, phosphorylated mTOR, total mTOR, phosphorylated GSK3 β , total GSK3 β , phosphorylated p70^{S6K}, total p70^{S6K}, phosphorylated ERK1/2, total ERK1/2, phosphorylated p38, total p38, phosphorylated JNKs and total JNKs were purchased from Cell Signaling Biotechnology (Beverly, MA). An antibody used to detect β -actin was from Santa Cruz Biotechnology (Santa Cruz, CA). The protein assay kit was from Bio-Rad (Hercules, CA).

Cell culture

MCF-7 and SK-BR-3 human breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured at 37°C with 5% CO₂ in DMEM or McCoy supplemented with 10% FBS, 2 mM L-glutamine, and 100 units/ml penicillin. Cells were cytogenetically tested and authenticated before being frozen. Each vial of frozen cells was thawed and maintained for about two months (16 passages).

In silico target identification

To identify potential binding proteins of UAB30 and Targretin, a shape similarity method from the PHASE module of Schrödinger's molecular modeling software package [17] was used to search for biological targets of UAB30 and Targretin based on their respective structures. The parameter of volume scoring was set as QSAR, meaning that the queries were used to calculate volume overlaps between atoms that have the same pharmacophore type (Acceptor, Donor, etc.) as defined for Phase QSAR models. The target libraries were obtained from the Protein Data Bank [18] and our in-house database. To provide more structural orientations for possible alignment, we set the maximum number of conformers per molecule in the generated library to 100 while retaining up to 10 conformers per rotatable bond. We filtered out conformers with a similarity score below 0.75 to obtain results of potential binding proteins of the queried compound.

Molecular modeling

The computer modeling of UAB30 and Targretin with Src was performed by using the Schrödinger Suite 2012 programs [19]. First, an X-ray diffraction structure of Src with a resolution of 2.4 Å (PDB ID 1BYG) [20] was obtained from the RCSB Protein Data Bank [18]. This structure was prepared under the standard procedures of the Protein Preparation Wizard in Schrödinger suite 2012. Hydrogen atoms were added consistent with a pH of 7 and all water molecules were removed. Finally, an ATP binding site-based receptor grid was generated for docking. UAB30 and Targretin were prepared using the LigPrep program of

Schrödinger for docking by default parameters. Docking of UAB30 and Targretin with Src was accomplished using the program Glide and default parameters under the extra precision (XP) mode to obtain the best-docked representative structures.

***In vitro* kinase assay**

The *in vitro* kinase assays were conducted in accordance with the instructions provided by Millipore. Active kinases were mixed with UAB30 or Targretin (0, 2, 10, 50 μ M) or 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2, 50 μ M) in reaction buffer [40 mM MOPS/NaOH (pH 7.0), 1 mM EDTA, 10 mM MnCl_2 , and 0.8 M L ammonium sulphate]. The mixture was incubated with 100 μ M substrate for 5 min at room temperature followed by incubation with 10 μ L of a ATP mixture [25 mM MgAc and 0.25 mM ATP-containing 10 μ Ci [γ - ^{32} P]ATP for 20 min at 30°C and then 25 μ L of reaction mixture were transferred onto P81 filter papers (Millipore Corp, Billerica, MA). The filter papers were washed with 0.75% phosphoric acid twice and with acetone once. The radioactive incorporation was determined using a scintillation counter.

***In vitro* invasion assay**

An *in vitro* invasion assay was performed using a 24-well transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA) as described previously [21]. The lower side of the filter was coated with 10 μ L of 0.5 mg/ml type I collagen, and the upper side was coated with 10 μ L of 0.5 mg/ml Matrigel (Bio-Rad). The lower compartment was filled with serum-free medium containing 0.1% BSA and each respective compound (50 μ M). Cells and each chemical (50 μ M) were placed in the upper part of the transwell plate. Cells were incubated for 24 h, fixed with methanol, and stained with hematoxylin for 10 min followed briefly by eosin staining. The invasive phenotypes were determined by microscopy (X400) and counting the cells that migrated to the lower side of the filter. Thirteen fields were counted for each filter and each sample was assayed in triplicate.

***In vitro* migration assay**

An *in vitro* migration assay was performed using a 24-well transwell unit with polycarbonate filters. Experimental procedures are the same as for the *in vitro* invasion assay described above except that the filter was not coated with Matrigel for the migration assay.

Wound migration assay

A wound migration assay was performed as previously described [21]. Briefly, cells were pretreated with mitomycin C (25 μ g/ml) for 30 min before an injury line was made. The injury line was created with a 2 mm wide tip on the cells plated in culture dishes at 90% confluence. After being rinsed with PBS, cells were allowed to migrate in complete medium containing UAB30 or Targretin (50 μ M); and photographs were taken (X40) at the indicated time points.

Gelatin zymography

A gelatin zymography assay was performed as previously described [22]. Cells were cultured in serum-free medium containing UAB30 or Targretin (50 μ M) for 1, 2, or 3 days. Conditioned media were collected and centrifuged at 3,000 g for 5 min to remove cellular debris. Equal amounts of conditioned media were determined using the *RC DC* protein assay (Bio-Rad, CA) and mixed with 23 Laemmli non-reducing sample buffer, incubated for 15 min at room temperature and then electrophoresed on 10% SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 twice for 30 min, rinsed 3 times for 30 min with a 50 mM Tris-HCl buffer (pH 7.6) containing 5 mM CaCl_2 , 0.02% Brij-35 and 0.2% sodium azide, and incubated overnight at 37°C. The gels were stained for 30 min with 0.5% Coomassie brilliant blue R-250 solution containing 10% acetic acid and 20% methanol and de-stained with 7.5% acetic acid solution containing 10% methanol. Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

Total RNA was obtained from cells treated or not treated with UAB30 or Targretin using an RNA isolation kit (GE Health care, PA) following the manufacturer's instructions. First-strand cDNAs were synthesized using the AmfiRivert II cDNA Synthesis Master Mix (GenDEPOT, TX) and specific primers for the human *MMP-9* or β -*actin* gene. Primers specific for *MMP-9* are (forward 5'-CTCCTCCCTTTCCTCCAGAACAGAA-3', reverse 5'-GGAGCCGCTCTCCAAGAAGCTT-3') and for β -actin (forward 5'-GGCATGGGTCAGAAGGATTTCG-3', reverse 5'-AGCACAGCCTGGATAGCAACG-3'), which produce amplified products of 520 and 287 bp, respectively. PCRs were run for 30 cycles using the following conditions: denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min.

Western blot analysis

Western blot analysis was performed as previously described [21]. Equal amounts of protein were separated by SDS/PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (GE Healthcare Biosciences, PA). Membranes were blocked with skim milk and incubated with appropriate primary antibodies overnight at 4°C. After washing with PBS containing 0.1% Tween 20, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody at a 1:5,000 dilution and the signal was detected with a chemiluminescence reagent (GE Healthcare Biosciences).

Lentiviral Infection

The lentiviral expression vectors, including *Gipz-shSRC* and packaging vectors, including *pMD2.0G* and *psPAX*, were purchased from Addgene Inc. (Cambridge, MA). To prepare Src viral particles, each viral vector and packaging vectors (*pMD2.0G* and *psPAX*) were transfected into 293T cells using JetPEI following the manufacturer's suggested protocols. The transfection medium was changed at 24 h after transfection and then cells were cultured for 36 h. The viral particles were harvested by filtration using a 0.45 mm syringe filter, then combined with 8 μ g/ml of polybrene (Millipore, Billerica, MA) and infected into 60%

confluent SK-BR-3 cells overnight. The cell culture medium was replaced with fresh complete growth medium for 24 h and then cells were selected with puromycin (1.5 µg/ml) for 48 h. The selected cells were used for experiments after confirming Src expression by Western blot.

Statistical analysis

All quantitative results are expressed as mean values $\pm$ S.D. Statistically significant differences were obtained using the Student's *t* test or by one-way ANOVA. A *p* value < 0.05 was considered to be statistically significant.

Results

Identification of UAB30 and Targretin protein targets using a shape similarity approach

To identify potential targets of UAB30 and Targretin, we first conducted *in silico* screening using a shape similarity approach. UAB30 and Targretin were screened against all the crystallized ligands available from the PDB (Table 1). We found potential targets that included protein kinase C (PKC), p38 and Src.

Src is a target of UAB30 and Targretin

To determine whether PKC, p38 or Src are potential targets of UAB30 (Fig. 1A) or Targretin (Fig. 1B) as suggested from the *in silico* screening, we assessed the effect of each compound on the respective kinase activity of each of these proteins. Both UAB30 and Targretin could inhibit Src kinase activity but did not affect p38 or PKC (Fig. 1C, D; Fig. S1). Screening results showed that UAB30 has a shape and pharmacophore similarity of 0.77 with 3-aryl-4-(1H-indole-3yl)-1, 5-dihydro-2H-pyrrole-2-ones, which is a reported c-Src inhibitor [23]. To better understand how UAB30 and Targretin interact with Src, we performed a computational docking study using the Glide docking program from Schrödinger Suite 2012. In the docked models, UAB30 or Targretin could bind at the ATP binding pocket of the tyrosine kinase domain of the Src C-terminal and several important hydrogen bonds were formed between UAB30 or Targretin and Src (Fig. 1E, F). This suggests that UAB30 and Targretin might be potential inhibitors against Src. Some images were generated with the UCSF Chimera program [24].

UAB30 or Targretin inhibits migration and invasion of MCF-7 and SK-BR-3 cells

Several studies reported that Src family kinases (SFKs) play key roles in cancer cell adhesion, motility, invasion and metastasis [25,26]. Metastasis is a multistep process in which tumor cells detach from the primary tumor and colonize in distant organs [27]. First, we determined the effects of UAB30 and Targretin on ER positive (MCF-7) and HER-2 overexpressing (SK-BR-3) breast cancer cells using a wound migration assay. Motility of MCF-7 (Fig. 2A, C, E, G) and SK-BR-3 (Fig. 2B, D, F, H) cells was greatly reduced by UAB30 or Targretin treatment (Fig. 2A, B, C, D). We also assessed migration using a transwell assay and found that UAB30 or Targretin inhibits migration of MCF-7 or SK-BR-3 cells (Fig. 2E, F, G, H). Next, we examined the effects of UAB30 or Targretin on the ability of both MCF-7 and SK-BR-3 cells to invade through a reconstituted basement membrane (Matrigel) in a transwell chamber. Results indicated that UAB30 or Targretin

significantly decreased the invasiveness of MCF-7 (Fig. 3A, B) or SK-BR-3 (Fig. 3C, D) cells compared with untreated control cells.

UAB30 or targretin decreases the expression and activity of MMP-9 in MCF-7 and SK-BR-3 cells

Increased invasion and migration of cancer cells is often associated with enhanced expression of MMP-2 and/or MMP-9, which can degrade type IV collagen, the major structural collagen of the basement membrane [28]. Therefore we determined the effect of UAB30 and Targretin on MMP-2 and MMP-9 expression in MCF-7 and SK-BR-3 cells. Results indicated that these compounds decreased the gelatinolytic activity of MMP-9 (92 kDa) but not that of MMP-2 (72 kDa) in MCF-7 (Fig. 4A) and SK-BR-3 (Fig. 4B) cells. Subsequently, we determined whether the decrease in MMP-9 caused by UAB30 or Targretin resulted from transcriptional inhibition. Semi-quantitative reverse transcription-PCR (RT-PCR) analysis revealed a decreased *MMP-9* mRNA level in MCF-7 and SK-BR-3 cells treated or not treated with UAB30 or Targretin of (Fig. 4C) cells. These results suggested that MMP-9 down-regulation might have resulted from transcriptional repression by UAB30 or Targretin at the mRNA level.

UAB30 or Targretin attenuates downstream PI3-K signaling but does not affect MAP kinases

We then determined whether Src downstream effector molecules are required for the inhibition of invasion and migration by UAB30 or Targretin. A number of studies reported that invasion is involved in Src-MAP kinase-dependent signaling [29] and Fyn, upstream of Src, regulates the Ras/PI3-K/AKT pathway in cancer [26]. Importantly, exposure of MCF-7 (Fig. 5A) or SK-BR-3 (Fig. 5B) cells to UAB30 or Targretin decreased the phosphorylation of PI3-K downstream molecules, including AKT, mTOR, GSK3 β and p70^{S6K} in both cell lines. However, MAP kinase signaling molecules in MCF-7 (Fig. 5C) or SK-BR-3 (Fig. 5D) cells were not affected by UAB30 or Targretin.

PP2 inhibits invasiveness of MCF7 and SK-BR-3 cells and Src shRNA transfection decreases the sensitivity of SK-BR-3 cells to UAB30 or Targretin

To verify the role of Src in breast cancer cell invasiveness, we used a pharmacological inhibitor of Src, PP2, and knocked down expression of Src by shRNA. PP2 inhibited the invasion of MCF7 and SK-BR-3 cells (Fig. 6A, B) and expression of Src shRNA (Fig. 6C) in SK-BR-3 cells also reduced invasiveness. Transducing Src shRNA caused excessive death of MCF-7 cells. Results indicated that the invasive ability of SK-BR-3 cells was decreased after transfection with Src shRNA compared with the mock-transfected group. SK-BR-3 cells transfected with Src shRNA were resistant to UAB30 and Targretin treatment (Fig. 6D, E). These results suggested that Src plays an important role in the sensitivity of SK-BR-3 cells to the inhibitory effects of UAB30 or Targretin.

Discussion

Off-target effects of many drugs can lead to unwanted side effects but also can be used to describe a drug's mechanism of action [30]. We conducted *in silico* screening by using a

shape similarity approach. From the computer modeling and our kinase assay results, we identified Src as a potential target of UAB30 and Targretin. Src is one of the oldest and most well-known oncogenes. Over the years, many reports have supported a key role of Src in various important cellular pathways, such as proliferation, differentiation, survival, metastasis, and angiogenesis [31,32]. Src kinase activity is highly up-regulated in breast cancer tissue and in invasive breast cancer cell lines. Moreover, Src family kinase inhibitor-treatment resulted in decreased motility and invasiveness in breast cancer cell lines [33]. UAB30 and Targretin have been used for breast cancer treatment as an RXR agonist. However, we suggest that Src is an off-target of UAB30 and Targretin based on the results of this study.

A number of studies have reported that invasion is involved in Src-MAP kinase-dependent signaling [29] and Fyn, upstream of Src, reportedly regulates the Ras/PI3-K/AKT pathway in cancer [26]. Haynes *et. al.* [34] reported that the Src family kinase specific inhibitor, PP2, suppressed 17 β -estradiol-induced PI3-K activation and also decreased 17 β -estradiol-induced AKT phosphorylation. Moreover, estrogen activated c-Src inducing the formation of an estrogen receptor and PI3-K regulatory subunit p85 complex. c-Src is a critical upstream regulator of the estrogen-stimulated PI3-K/AKT/eNOS pathway. Interestingly, UAB30 and Targretin did not affect the MAP kinase pathway but specifically down-regulated the protein expression of the Src-PI3-K/AKT signaling pathway. Our data strongly suggested that Src is a key target molecule of these RXR agonists to regulate the Src/PI3-K/AKT pathway in human breast cancer cells.

Activation of the PI3-K/AKT pathway is an important regulatory function in MMP expression. *MMP* promoters have diverse regulatory motifs recognized by numerous proteins such as AP-1, NF- κ B, and S6K. A novel quinazolinone reportedly inhibited prostate cancer metastasis through the MAP kinase, AKT, NF- κ B and AP-1 signaling pathways [35]. One report indicated that the quinazolinone derivative, NJ-56, abrogated the PI3-K/AKT/mTOR signaling pathway leading to decreased protein synthesis by dephosphorylating the translation initiation factors, including S6 ribosomal protein. Additionally NJ-56 reduced NF- κ B-mediated transcription of MMPs through attenuated PI3-K/AKT activation, resulting in inhibition of colon cancer cell metastasis [36]. These reports suggested that the alteration of *MMP-9* mRNA levels and activities by RXR agonists might be regulated by the AKT/mTOR/S6K pathways. However we need further investigations to determine the detailed molecular mechanisms explaining the regulation of MMP-9 by RXR agonists, UAB30 and Targretin, in breast cancer cells.

We found that UAB30 and Targretin decreased migration and invasion of human breast cancer cells and the decrease was associated with reduced MMP-9 activity and expression, which might be regulated by Src. Additionally, knocking down Src expression in SK-BR-3 cells also decreased their invasive ability. Moreover, repressing Src expression in SK-BR-3 cells caused them to lose their sensitivity to the inhibitory effects of UAB30 and Targretin, indicating that the antitumor activities of these RXR-agonists could arise mainly through modulation of Src and its downstream pathways. In summary, we have shown that UAB30 or Targretin decreases migration and reduces invasion of MCF-7 and SK-BR-3 breast cancer

cells by targeting Src and suppressing its downstream signaling. Inhibition of these signaling pathways also led to decreased MMP-9 expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

UAB30 or Targretin decreases Src kinase activity. Chemical structure of UAB30 (A) or Targretin (B). UAB30 (C) or Targretin (D) inhibits Src kinase activity. Active Src (10 ng) was mixed with UAB30 or Targretin (0, 2, 10, 50 μM) or PP2 (Src inhibitor, 50 μM) and then incubated with a [γ - ^{32} P] ATP mixture. The radioactive incorporation was determined using a scintillation counter. Modeling of UAB30 (E) or Targretin (F) with Src. The docking conformation of UAB30 and Targretin in the ATP-binding pocket of Src is shown.

Figure 2. UAB30 or Targretin inhibits migration of MCF-7 or SK-BR-3 cells. The effect of UAB30 or Targretin on migration of MCF-7 or SK-BR-3 cells was assessed by a wound-healing assay. MCF-7 (A, C) or SK-BR-3 (B, D) cells were treated with mitomycin C (25 μ g/ml) for 30 min and then an injury line was made on the confluent monolayer of cells. Cells were incubated in complete medium containing 50 μ M UAB30 or Targretin. Cell motility was examined by light microscope (X40) at the indicated time points. Width of the injury line from 3 independent experiments was measured and plotted. The effect of UAB30 or

Targretin (50 μ M) on the migration of MCF-7 (E, G) or SK-BR-3 (F, H) cells was assessed by a transwell migration assay as described in Materials and Methods. All data are shown as means $\pm$ S.D. (N = 5) and the asterisk (*) indicates a significant ($p < 0.05$) difference compared to untreated control.

Figure 3.

UAB30 or Targretin inhibits invasion of MCF-7 or SK-BR-3 cells. The effect of UAB30 or Targretin (50 μ M) on invasion of MCF-7 (A, B) or SK-BR-3 (C, D) cells was assessed by a transwell invasion assay as described in Materials and Methods. Data are shown as means $\pm$ S.D. (N = 5) and the asterisk (*) indicates a significant ($p < 0.05$) difference compared to untreated control.

Figure 4.

UAB30 or Targretin inhibits matrix metalloproteinase (MMP)-9 activity in MCF-7 or SK-BR-3 cells. UAB30 or Targretin inhibits MMP-9 activity in MCF-7 (A) or SK-BR-3 (B) cells. Conditioned media were prepared from MCF-7 and SK-BR-3 cells treated or not treated with UAB30 or Targretin (50 μ M) for the indicated time. MMP activity was measured by gelatin zymography as described in Materials and Methods. UAB30 or Targretin inhibits *MMP-9* mRNA expression in MCF-7 and SK-BR-3 (C) cells. mRNA was prepared from MCF-7 or SK-BR-3 cells treated or not treated with UAB30 or Targretin (50

μM) for the indicated time. mRNA levels were measured by RT-PCR as described in Materials and Methods. The housekeeping *β-actin* gene served as a loading control.

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Figure 5.

UAB30 or Targretin inhibits phosphorylation of the AKT pathway but does not affect phosphorylation of mitogen activated protein (MAP) kinases in MCF-7 or SK-BR-3 cells. UAB30 or Targretin inhibits phosphorylation of AKT/mTOR/GSK3 β /p70^{S6K} in MCF-7 (A) or SK-BR-3 (B) cells. UAB30 or Targretin does not affect MAP kinase activity in MCF-7 (C) or SK-BR-3 (D) cells. The proteins were prepared from MCF-7 and SK-BR-3 cells treated or not treated with UAB30 or Targretin (50 μ M) for 24 h. Western blot analysis was

performed using specific antibodies and representative blots are shown from triplicate experiments that gave similar results.

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Figure 6.

Inhibition of Src by PP2 or shRNA impairs invasiveness. In the presence with 10 μ M PP2, a known inhibitor of Src, a transwell invasion assay using MCF7 or SK-BR-3 cells was performed as described in Materials and Methods (A, B). *shRNA* impairs Src expression in SK-BR-3 cells (C). The proteins were prepared and analyzed by Western blot. In the presence of 50 μ M UAB30 or Targretin, a transwell invasion assay was performed as described in Materials and Methods using mock- or Src-*shRNA* transfected cells (D, E). Data

are shown as means $\pm$ S.D. (N = 5) and the asterisk (*) indicates a significant ($p < 0.05$) difference compared to untreated control.

Table 1

Potential kinase targets of 9-cis-UAB30 and Targretin

9-cis-UAB30		Targretin	
Target	Shape similarity	Target	Shape similarity
TGF-beta receptor type I	0.81	peroxisome proliferator activated receptor γ	0.880
protein kinase C	0.79	tumor necrosis factor alpha	0.808
cyclin-dependent kinase 1 (CDK1)	0.79	p-selectin	0.801
p38	0.77	cytochrome P450 17A1	0.764
Src	0.77	androgen receptor (AR)	0.763