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Small molecule inhibition of arylamine *N*-acetyltransferase Type I inhibits proliferation and invasiveness of MDA-MB-231 breast cancer cells

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ABSTRACT

Arylamine *N*-acetyltransferase 1 is a phase II metabolizing enzyme that has been associated with certain breast cancer subtypes. While it has been linked to breast cancer risk because of its role in the metabolic activation and detoxification of carcinogens, recent studies have suggested it may be important in cell growth and survival. To address the possible importance of NAT1 in breast cancer, we have used a novel small molecule inhibitor (Rhod-o-hp) of the enzyme to examine growth and invasion of the breast adenocarcinoma line MDA-MB-231. The inhibitor significantly reduced cell growth by increasing the percent of cells in G2/M phase of the cell cycle. Rhod-o-hp also reduced the ability of the MDA-MB-231 cells to grow in soft agar. Using an *in vitro* invasion assay, the inhibitor significantly reduced the invasiveness of the cells. To test whether this effect was due to inhibition of NAT1, the enzyme was knocked down using a lentivirus-based shRNA approach and invasion potential was significantly reduced. Taken together, the results of this study demonstrate that NAT1 activity may be important in breast cancer growth and metastasis. The study suggests that NAT1 is a novel target for breast cancer treatment.

Introduction

The arylamine *N*-acetyltransferases (NATs) are a family of conjugating enzymes that were originally linked to cancer risk because of their ability to metabolically activate many arylamine and heterocyclic amine carcinogens [1]. In humans, the NATs are encoded by 2 closely related genes located on chromosome 8. Arylamine *N*-acetyltransferase II (NAT2) is primarily found in the liver and gastrointestinal tract while arylamine *N*-acetyltransferase I (NAT1) is more widely distributed [2]. Both the NAT1 and NAT2 genes are genetically polymorphic and many studies have suggested that they are low penetrance genes that affect cancer risk [3]. In breast cancer, NAT2 'rapid' genotype has been linked with increased cancer risk, and the link is strongest in combination with smoking history [4–8].

In 2000, Perou and associates reported on the gene expression profiles for a number of different breast cancer subtypes [9]. They showed that NAT1 clustered with a number of genes that included the estrogen receptor (ER), GATA binding protein and X-box binding protein. Interestingly, NAT1 expression is 17-fold greater in vessels microdissected from breast tumors compared to vessels from normal tissue [10].

Despite several associations with cancer, NAT1 has not been shown to influence growth or survival other than in a non-transformed breast cell-line where elevated NAT1 appeared to provide a growth advantage [11]. Until recently, it was difficult to address the importance of NAT1 in cancer as there were no useful small molecule inhibitors available. A screen of a library of 5000 compounds against five different recombinant NATs from prokaryote and eukaryote sources identified several specific inhibitors for NAT1 that were based on the structure of rhodanine [12]. One of these, (Z)-5-(2′-hydroxybenzylidene)-2-thioxothiazolidin-4-one, demonstrated potent inhibition of human NAT1 (IC_{50} = 1.1 μM). Docking studies suggested that this compound interacted with the hydrophobic pocket of the active site of the enzyme. The crystal structure of this compound (Rhod-o-hp) has suggested that it may interact with adjacent molecules forming several weak bonds such as phi bonds and hydrogen bonds [13].

In the present study, we have used the small molecule inhibitor Rhod-o-hp to decrease NAT1 activity in the breast carcinoma cell-line MDA-MB-231 in order to study what effect, if any, loss of NAT1 activity has on cell function. In particular, we examined the effect of NAT1 inhibition on MDA-MB-231 cell growth and invasion.

Materials and methods

Reagents. Rhod-o-hp was a kind gift from Prof. Jose Sordo, Universidade de Santiago de Compostela. The synthesis and

Abbreviations: NAT1, arylamine *N*-acetyltransferase 1; Rhod-o-hp, 5-(2-hydroxybenzylidene)rhodanine; shRNA, small hairpin RNA interference.

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purification of the compound has been reported previously [13]. All other chemicals were obtained from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, USA) and were of the highest purity available.

Cell culture. Human breast cancer cell-line, MDA-MB-231, was obtained from Ms. Damara McAndrew, University of Queensland, Australia. Cells were cultured in DMEM (Invitrogen, Melbourne, VIC, Australia) supplemented with 10% fetal bovine serum (Invitrogen), 10 μM *p*-aminobenzoic acid (PABA) and penicillin/streptomycin, and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

NAT1 inhibition in cultured cells. Cells were seeded at 1.5×10^5 cell per well in 24-well plates. After treating cells with increasing concentrations of Rhod-o-hp for 24 h, the culture medium was collected and *N*-acetyl-PABA quantified by HPLC as described elsewhere [14].

Recombinant human NAT1. The protein coding sequence of human wild-type *NAT1* was cloned into the bacterial expression vector pET-28a (Novagen, Merck Australia, Kilsyth, VIC, Australia) in-frame with the N-terminal His-tag and introduced into the *E. coli* strain BL21-CodonPlus(DE3)-RP (Stratagene, Agilent Technologies, Forest Hill, VIC, Australia). Bacteria were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside overnight at 18 °C. His-tagged protein was purified using TALON metal affinity resin (Clontech Laboratories Inc., Mountain View, CA, USA) and eluted with 250 mM imidazole. Recombinant protein was then dialyzed against NAT1 assay buffer (20 mM Tris (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol) overnight at 4 °C to remove imidazole. Protein concentration was determined by the method of Bradford [15].

NAT1 activity assay. Recombinant NAT1 (15 ng) was incubated with PABA (0.42 mM) and acetyl Coenzyme A (1.1 mM) in the presence of Rhod-o-hp (0–300 μM) or vehicle (DMSO) for 10 min. NAT1 activity was determined as previously described [17]. Reactions were performed under linear conditions with respect to time and protein concentration.

Growth curves. Cells were seeded at 1×10^4 cell per well in 96-well plates and treated with Rhod-o-hp (0–300 μM). Culture medium containing fresh Rhod-o-hp was replaced daily. Cell proliferation was measured over 6 days using a CellTiter96® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, USA).

Soft-agar assay. Cells were suspended in 0.3% agar supplemented with increasing Rhod-o-hp concentrations and layered onto 0.6% base agar, also supplemented with Rhod-o-hp, in 6-well plates (3000 cells/well). This was then covered with culture medium containing Rhod-o-hp, which was replaced daily. After 21 days, colonies were stained with 0.005% crystal violet for 1 h and colonies counted using QuantityOne Software (Bio-Rad, Hercules, CA, USA).

Invasion assay. Transwell supports with an 8 μm pore (Corning, Lowell, MA, USA) were layered with (test) or without (control) 10–15 μg Geltrex (Invitrogen) and cells suspended in culture medium supplemented with 0.2% fetal bovine serum and Rhod-o-hp were seeded onto the inserts. The inserts were then placed into a 24-well plate containing culture medium supplemented with 10% fetal bovine serum and Rhod-o-hp. After 24 h, the membrane inserts were fixed and stained with toluidine. The number of cells that transmigrated through the matrix was counted and the percent cell invasion (%CI) was calculated as:

$$\%\mathrm{CI} = 100 \times \frac{(\text{Number of cells invaded through the Geltrex insert})}{(\text{Number of cells migrated through control insert})}$$

shRNA lentivirus system. The RNAi sequence used to down-regulate human NAT1 was predicted by Sfold software (NAT1 RNAi sequence; 5′-GGGAACAGTACATTCCAAA-3′; scrambled RNAi sequence; 5′-GGAATCTCATTCGATGCAT-3′). The RNAi sequences were cloned into pLL3.7 Lenti-lox vector as described online by Dillon et al. (http://web.mit.edu/jacks-lab/protocols/pll37cloning.htm) and lentivirus

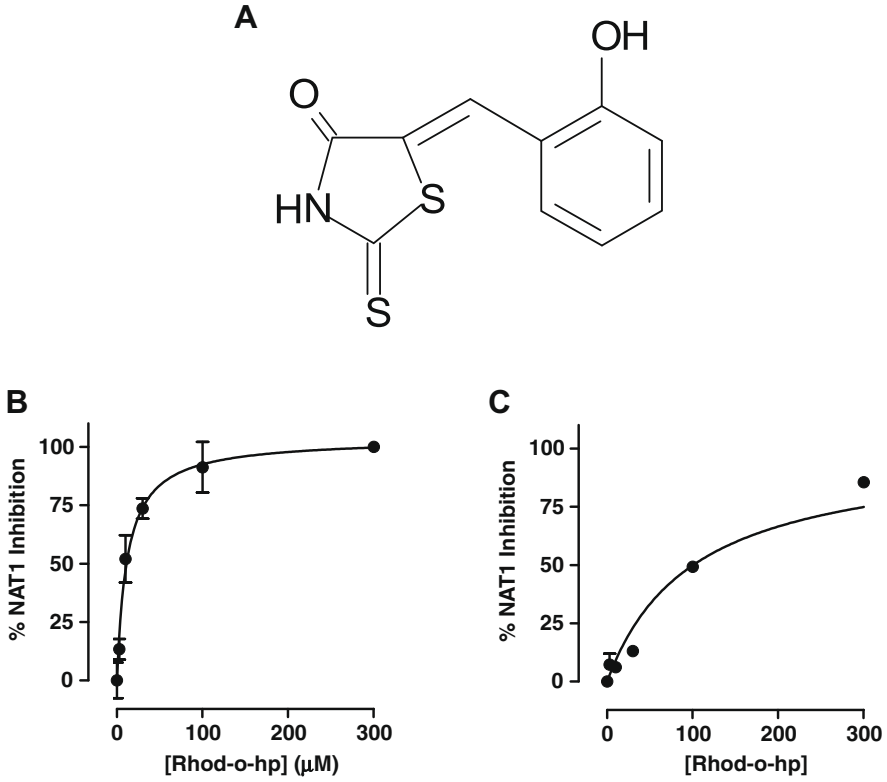

Fig. 1. Small molecule inhibitor of NAT1, Rhod-o-hp, inhibits NAT1 at micromolar concentrations in a dose-dependent manner. (A) Chemical structure of Rhod-o-hp. (B) *In vitro* inhibition of recombinant human NAT1. Results are presented as means ± SEM, $n = 3$. (C) Inhibition of NAT1 by Rhod-o-hp in cultured human MDA-MB-231 cells. Results are presented as means ± SEM, $n = 3$.

production was performed as described elsewhere [16] with minor modifications. Briefly, pLL3.7 and packaging vectors were co-transfected into 293T cells and the supernatant was collected after 36 h. Supernatant was centrifuged at 4 °C and filtered through a 0.45 μm filter membrane. Virus particles were concentrated using 100,000 MWCO polyethersulfone spin columns (Vivaspin 20; Sartorius Stedim Biotech GmbH, Goettingen, Germany) according to the manufacturer's instructions. MDA-MB-231 cells were transduced with concentrated lentivirus particles in the presence of 6 μg/ml polybrene for 20 h. Following transduction, cells were expanded and experiments performed within 5 cell passages. Transduction efficiency was determined by quantifying cells positive for EGFP expression by flow cytometry.

Data analysis and statistics. Data are expressed as means ± SEM. Statistical comparisons between different treatments were assessed by Student's *t*-tests or one-way ANOVA assuming significance at a p value of 0.05 or less using Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

Results

NAT1 activity is inhibited by Rhod-o-hp

Rhod-o-hp is a 5-benzylidenerhodanine derivative that contains a hydroxyl group in the ortho position of the benzene ring (Fig. 1A). Using recombinant NAT1 and PABA as substrate, Rhod-o-hp inhib-

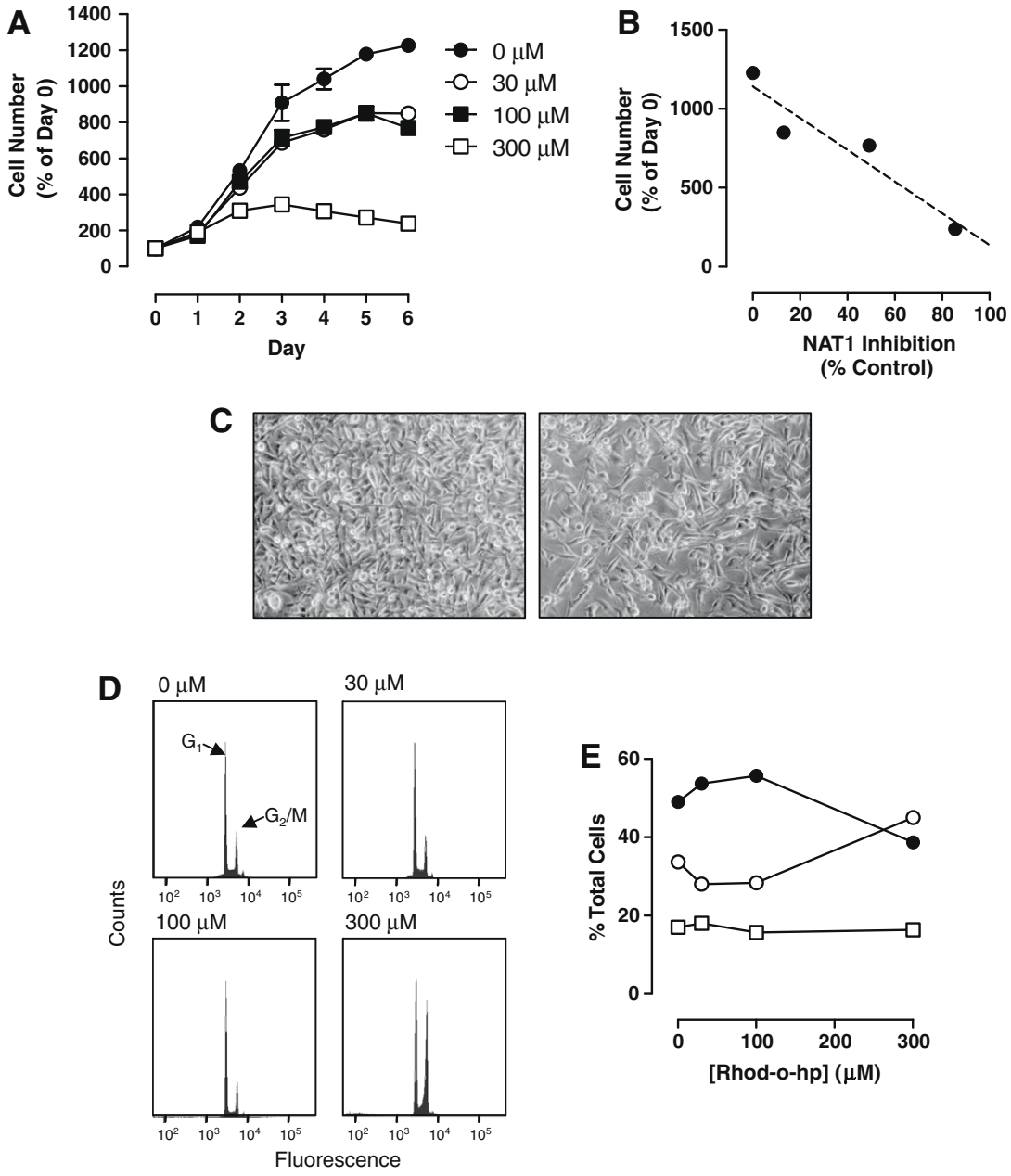

Fig. 2. Rhod-o-hp inhibits proliferation of MDA-MB-231 cells in culture. (A) Cells were treated with increasing concentrations of Rhod-o-hp and cell growth was determined up to 6 days. Results are presented as means ± SEM, $n = 4$. (B) Correlation between the *in vivo* inhibition of NAT1 and proliferation. The correlation coefficient (r^2) for linear regression was 0.9 ($p < 0.05$). (C) Morphology of cells 6 days following treatment with vehicle (left panel) or 300 μM Rhod-o-hp (right panel). (D) Cell cycle analysis by flow cytometry of MDA-MB-231 cells after 4 days treatment with increasing concentrations of Rhod-o-hp. (E) Percentage of cells in each phase of the cell cycle following 4 days treatment with Rhod-o-hp. Results are presented as means ± SEM, $n = 3$.

ited NAT1 enzyme activity in a concentration-dependent manner (Fig. 1B). The estimated IC_{50} was 12.1 ± 2.0 μM, which is slightly higher than reported values [12]. This may be due to different assay conditions. At 100 μM, Rhod-o-hp inhibited NAT1 activity by more than 95%. To test whether Rhod-o-hp could inhibit NAT1 in intact cells, MDA-MB-231 cells were incubated with PABA in the presence of increasing concentrations of Rhod-o-hp and *N*-acetylated PABA in the culture medium was measured after 24 h. Again, a concentration-dependent inhibition of NAT1 was observed (Fig. 1C). However, the IC_{50} was much greater (101 ± 11 μM), which may be due to limited uptake of the compound or its degradation by the cells. At 300 μM Rhod-o-hp, NAT1 activity was inhibited by more than 85%.

Rhod-o-hp inhibits MDA-MB-231 proliferation by blocking cells in G_2/M

When MDA-MB-231 cells were treated with Rhod-o-hp, cell proliferation over 6 days decreased in a concentration-dependent manner (Fig. 2A). At the highest concentration, cell growth was completely inhibited after 3 days. There was a high correlation between the inhibition of NAT1 and the inhibition of cell growth (Fig. 2B, $r^2 = 0.9$). The morphology of the treated cells (Fig. 2C, right panel) was normal when compared to untreated cells (Fig. 2C, left panel) although the treated cells were less confluent. No evidence of cell rounding or apoptotic bodies was seen with drug treatment suggesting that the cells were quiescent as opposed to dead. To test this, cells were treated with drug for 4 days and then cell cycle analysis was performed. As the drug concentration increased, there was an increase in the percentage of cells in G_2/M phase of the cell cycle (Fig. 2C). This was accompanied by a decrease in the percentage of cells in G_1 (Fig. 1D).

Rhod-o-hp inhibits MDA-MB-231 growth in soft agar

The ability of cells to grow in an anchorage-independent manner is a hallmark of transformation and often correlates with tumor formation *in vivo* [17,18]. A soft-agar assay was performed to investigate whether Rhod-o-hp affected anchorage-independent growth of MDA-MB-231 cells (Fig. 3A). The drug decreased colony

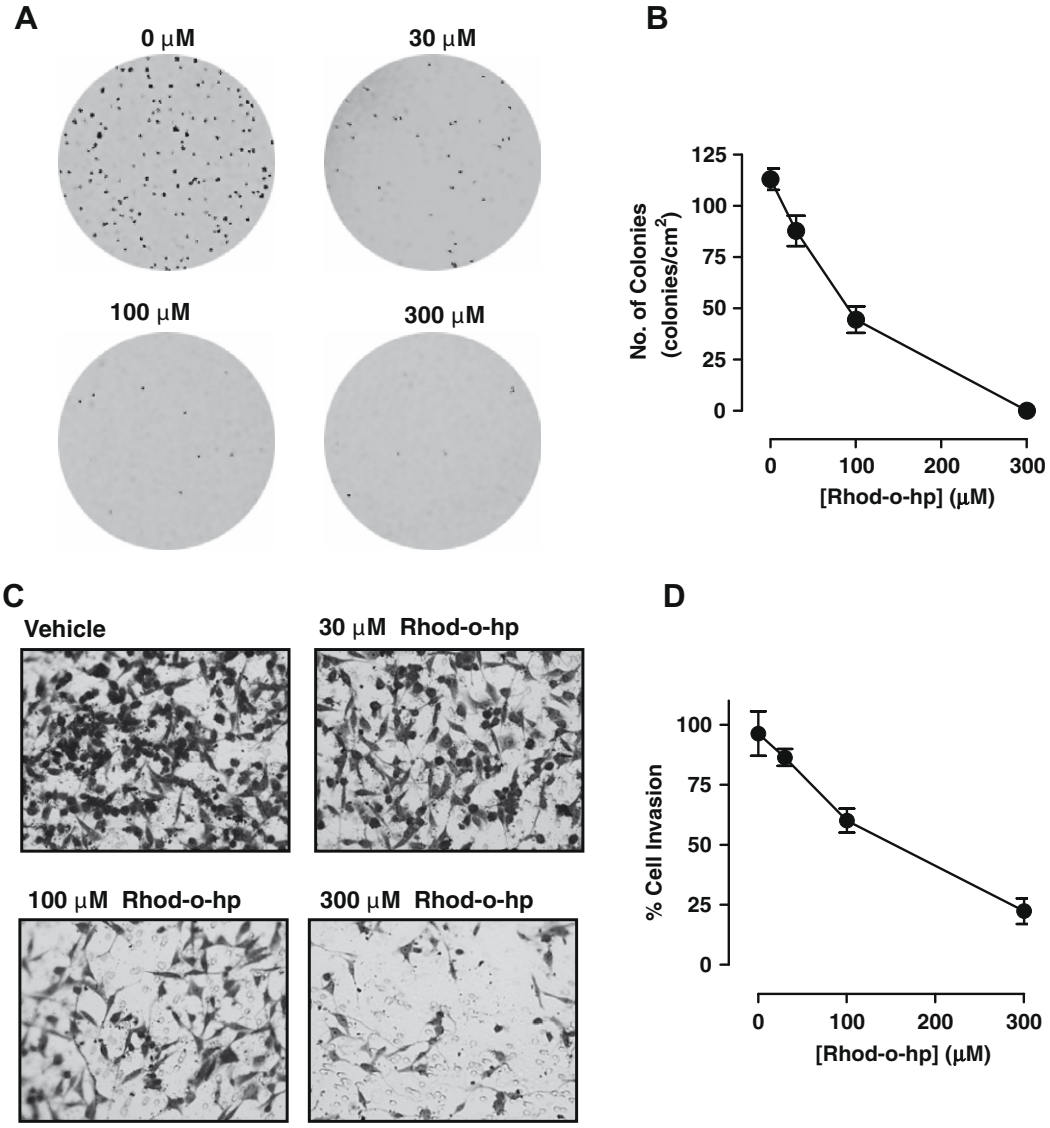

Fig. 3. Rhod-o-hp inhibits anchorage-independent growth and invasion of MDA-MB-231 cells. (A) Rhod-o-hp treatment of cells resulted in a dose-dependent reduction in soft agar colony formation. (B) Quantification of the soft-agar assay. Results are presented as means ± SEM, $n = 3$. (C) Representative photographs of cells from an invasion assay. After 24 h, the membrane inserts were fixed and stained with toluidine. (D) Quantification of the invasion assay. The number of cells that transmigrated through the matrix was counted and the percent cell invasion was calculated relative to the number of cells in the absence of matrix. Results are presented as means ± SEM, $n = 3$.

formation from more than 100/cm^2 in untreated cells to less than 2/cm^2 at 300 μM (Fig. 3B).

Rhod-o-hp inhibits MDA-MB-231 cell invasion

A loss of the ability to grow in soft agar has been associated with a decrease in invasion potential of transformed cells [19–21]. Invasion is critical for formation of metastases and the spreading of a cancer *in vivo*. To test whether Rhod-o-hp altered MDA-MB-231 invasiveness, we used a transwell layered with Geltrex, a combination of extracellular matrix components that support cell adhesion and proliferation. Cell invasion was quantified as the number of cells that could traverse the chamber in 24 h. Rhod-o-hp caused a dose-dependent decrease in cell invasion, which was evident when cells were stained (Fig. 3C). Quantification of the cells in the chamber showed a decrease of approximately 75% at 300 μM drug (Fig. 3D).

NAT1 shRNA inhibits MDA-MB-231 cell invasion

Although Rhod-o-hp is an inhibitor of NAT1 in intact cells, its specificity is unknown. Therefore, the effect of the drug on cell growth and invasion could result from cellular perturbations unrelated to NAT1. If inhibition of NAT1 was responsible for the observed changes, then inhibition of the enzyme by an independent method should result in similar changes. To address this, we used a lentivirus system to express shRNA directed against NAT1 mRNA to inhibit the enzyme and then determined the effect on cell invasion. MDA-MB-231 cells were infected with the lentivirus, which co-expressed EGFP as a marker of infection, with an efficiency of 82% for control cells (scrambled sequence) and 75% for NAT1 shRNA-treated cells (Fig. 4A). NAT1 activity was significantly decreased by 74% in NAT1 shRNA-treated cells (Fig. 4B). Moreover, significantly less invasion was seen for the NAT1 shRNA-treated cells (Fig. 4C). Quantitatively, this amounted to a 50% decrease in the number of cells that traversed the transwell (Fig. 4D). These results show that selective knock-down of NAT1 with shRNA also inhibited the invasiveness of MDA-MB-231 cells and supports the finding that inhibition of NAT1 with Rhod-o-hp was responsible for the effect of the drug.

Discussion

Rhod-o-hp is a small molecule inhibitor of NAT1 first identified in a library screen of compounds against several recombinant NATs [12]. In solution, it inhibits NAT1 with an IC_{50} in the low micromo-

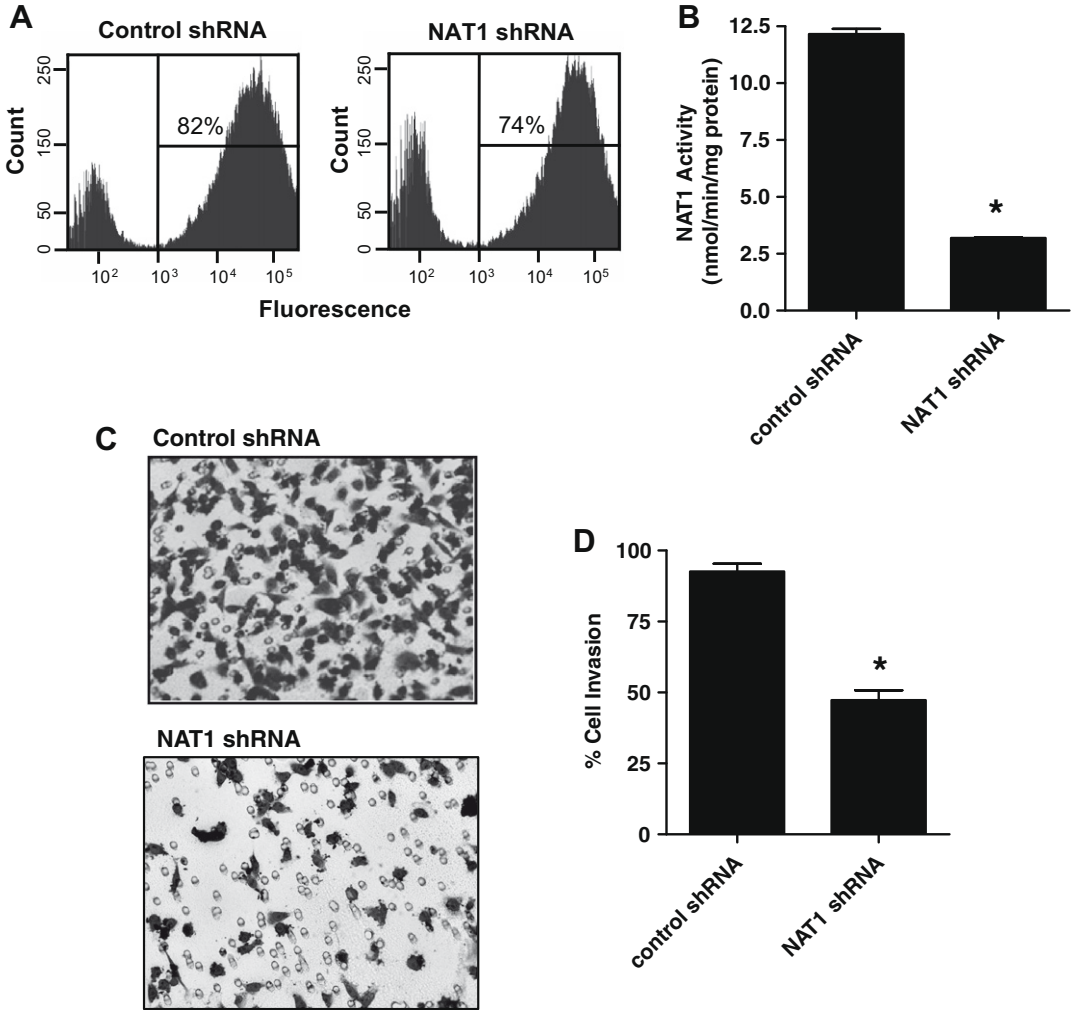

Fig. 4. Inhibition of MDA-MB-231 cells by NAT1-directed shRNA. (A) MDA-MB-231 cells were transduced with lentivirus that carried either a scrambled shRNA sequence (control shRNA) or NAT1-directed shRNA (NAT1 shRNA) sequence. The pLL3.7 Lenti-lox lentivirus system allows simultaneous expression of shRNA and EGFP in the transduced cells. Lentivirus transduction efficiencies of both control and NAT1 shRNAs were determined by scoring EGFP positive cells by flow cytometry. (B) The lentivirus-based shRNA achieved a 74% knock-down of NAT1 in MDA-MB-231 cells. Results are presented as means ± SEM, $n = 3$. Asterisk denotes a significant difference ($p < 0.05$). (C) The invasiveness of MDA-MB-231 cells was greatly reduced following NAT1 knock-down. (D) Quantification of cell invasion showed a significant reduction in the number of cells that invaded through Geltrex following NAT1 knock-down. Results are presented as means ± SEM, $n = 3$. Asterisk denotes a significant difference ($p < 0.05$).

lar range. However, it is somewhat less effective in whole cells suggesting it is degraded or poorly taken up. Nevertheless, it inhibited NAT1 activity in a dose-dependent manner that correlated closely with its ability to inhibit cell proliferation and to inhibit growth in soft agar. Cell toxicity was not evident. Rhod-o-hp also significantly affected the ability of MDA-MB-231 cells to invade using an *in vitro* invasion assay. These effects of the drug are most likely due to NAT1 inhibition because specific NAT1 knock-down using shRNA technology produced similar effects.

Previously, Adam et al. [11] demonstrated a similar relationship between NAT1 activity and cell proliferation in HB4a cells. However, they described changes following an increase in NAT1 expression as opposed to a decrease as reported here. HB4a cells are an immortalized human breast epithelial cell-line that exhibits cell contact inhibition and they fail to form colonies in soft agar. When these cells were stably transfected with NAT1, they not only showed more rapid proliferation, but also appeared to grow independent of growth factors (in 0.5% serum). This work reinforces the findings in the present study, which proposes that NAT1 has a role in cell growth. It appears that unregulated high activity of NAT1 can drive proliferation and survival while low activity results in a phenotype more closely aligned to non-transformed cells in that they exhibit cell–cell contact inhibition and lack anchorage-independent growth. This may also explain, in part, why the murine homolog of NAT1 (Nat2) can be successfully knocked out in mice with little phenotypic change [22,23] while over-expression of NAT1 in mice appeared to be embryonic lethal [24].

Invasion is a hallmark of aggressive tumors and a characteristic of all metastatic cancers. Soft-agar assays can be predictive of invasion potential, although an invasion assay that requires cells to migrate through an extracellular matrix more accurately mimics cell spreading *in vivo* [25]. Treatment of MDA-MB-231 cells with Rhod-o-hp significantly reduced their invasion capacity. This was most likely due to inhibition of NAT1 because a similar result was observed when NAT1 was knocked down with shRNA. Recently, cisplatin has been shown to target NAT1 in cultured cells [26]. It is a potent irreversible inhibitor of the enzyme and it was proposed that NAT1 may be an important target for this drug *in vivo*.

In conclusion, given the results reported herein, there is growing evidence that NAT1 may be a novel therapeutic target in breast cancer. Further studies are required to identify the molecular pathway that links NAT1 expression to cell growth and invasion.

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