**TOP2A is overexpressed and is a therapeutic target for adrenocortical carcinoma**

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**Abstract**

Adrenocortical carcinoma (ACC) is a rare but aggressive malignancy with no effective therapy for patients with unresectable disease. The aim of the current study was i) to evaluate TOP2A expression and function in human adrenocortical neoplasm and ACC cells and ii) to determine the anticancer activity of agents that target TOP2A. TOP2A mRNA and protein expression levels were evaluated in 112 adrenocortical tissue samples (21 normal adrenal cortex, 80 benign adrenocortical tumors, and 11 ACCs). In vitro siRNA knockdown of TOP2A in ACC cell lines (NCI-H295R and SW13) was used to determine its effect on cellular proliferation, cell cycle, anchorage-independent growth, and cellular invasion. We screened 14 TOP2A inhibitors for their anticancer activity in ACC cells. TOP2A mRNA and protein expression was significantly higher in ACC than in benign and normal adrenocortical tissue samples ($P<0.05$). Knockdown of TOP2A gene expression in ACC cell lines significantly decreased cell proliferation, anchorage-independent growth, and invasion ($P<0.05$). A screening assay in NCI-H295R cells showed that 11 of 14 TOP2A inhibitors had antiproliferative activity, 5 of the 14 TOP2A inhibitors had a higher antiproliferative activity than mitotane, and aclarubicin was the agent with the highest activity. Aclarubicin was validated to significantly decrease proliferation and tumor spheroid size in both NCI-H295R and SW13 ACC cell lines ($P<0.05$). Our results suggest that TOP2A is overexpressed in ACC, regulates cellular proliferation and invasion in ACC cells, and is an attractive target for ACC therapy. Of the TOP2A inhibitors screened, aclarubicin is a good candidate agent to test in future clinical trials for patients with locally advanced and metastatic ACC.

**Key Words**
- adrenocortical carcinoma
- TOP2A
- TOP2 inhibitors
- aclarubicin
- invasion

**Introduction**

Adrenocortical carcinoma (ACC) is a rare but aggressive malignancy of the adrenal cortex (Favia et al. 1995, Wajchenberg et al. 2000, Icard et al. 2001, Bilimoria et al. 2008). Prognosis is poor in patients with locally advanced and metastatic ACC with 5-year survival rate of $<10\%$ (Allolio & Fassnacht 2006, Kebebew et al. 2006, Bilimoria et al. 2008). Therefore, there is a significant need for the identification of new therapeutic options that may be effective in patients with ACC.

Genome-wide gene expression profiling analysis has been used to identify dysregulated gene expression associated with ACC (Giordano et al. 2003, Velazquez-Fernandez et al. 2005, Lombardi et al. 2006, Slater et al. 2006, Fernandez-Ranvier et al. 2008). One of the genes
consistently observed to be overexpressed in ACC is topoisomerase alpha 2 (TOP2A; Giordano et al. 2003, Fernandez-Ranvier et al. 2008, Dawany et al. 2011). TOP2A encodes a DNA topoisomerase that controls and alters the topological states of DNA during transcription and is thus involved in processes such as chromosome condensation and chromatid separation. TOP2A has been shown to be a marker of proliferation, aggressive disease, and chemotherapy resistance in a variety of human cancers (Tretiakova et al. 2006, Kosari et al. 2008, Bedard et al. 2009, Coss et al. 2009, Desmedt et al. 2011, Malhotra et al. 2011). Targeting TOP2A with a variety of agents having TOP2A inhibitor activity, such as the anthracycline drugs, has emerged as an attractive strategy for cancer therapy and has led to clinical trials to test the efficacy of these compounds (Yamada et al. 1980, Karanes et al. 1983, Schutte et al. 1983, Kern et al. 1998, Nitiss 2009, Song et al. 2011, Wang et al. 2011).

In this study, we examined the expression and function of TOP2A in human adrenocortical tissue samples and ACC cells. We found that TOP2A was overexpressed in ACCs. Using siRNA knockdown of TOP2A in ACC cell lines, we found that it regulates cellular proliferation and invasion. Given the consistent overexpression of TOP2A in ACC, we tested 14 TOP2A inhibitors for their antiproliferative effect and found that 11 of the 14 compounds had good efficacy and of these aclorubicin had the most potent anticancer activity.

Materials and methods

Tissue specimens

Adrenal tissue samples were collected at surgery, snap frozen, and stored at −80 °C. In this study, 112 human adrenocortical tissue specimens were analyzed (21 normal adrenal cortex, 80 benign adrenocortical tumors, and 11 ACCs). The clinical protocol was approved by the institutional review board and written informed consent was obtained. The inclusion criterion for diagnosis of ACC was made by the presence of Weiss score ≥3.

Cell culture, reagents, and siRNA transfection

The NCI-H295R and SW13 ACC cell lines (ATCC, Rockville, MD, USA) were grown and maintained in DMEM media supplemented with 1% insulin transferrin selenium (BD Biosciences, San Jose, CA, USA) and 2.5% Nu-Serum 1 (BD Biosciences) in a standard humidified incubator at 37 °C in a 5% CO2 atmosphere. A nonspecific negative control siRNA (AM4613) and TOP2A-specific siRNAs at a final concentration of 90 nM were used (si#1; s14308 and si#3; s14309, Applied Biosystems).

Immunohistochemistry

Primary anti-TOP2A mouse MAB was used (Enzo Life Sciences, Ann Arbor, MI, USA; ADI-KAM-CC21) at a 2.5 μg/ml dilution overnight at 4 °C. The biotinylated secondary antibody was used 1:1000 dilution (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. TOP2A immunostaining was evaluated by light microscopy (Nikon, Tokyo, Japan) and images were scanned at 20× and 40× magnifications.

RNA preparation, RT, and real-time quantitative PCR

RNA was extracted using the TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Inc.). RNA quantity and quality were assessed using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) respectively. Total RNA (200–500 ng) was reverse transcribed using a High-Capacity Reverse Transcription cDNA kit and cDNA was amplified according to the manufacturer’s instructions (Applied Biosystems). The PCR primers and probes for TOP2A (Hs_00180383_m1), GAPDH (Hs_99999905_m1), TOP1 (Hs00243257_m1), and TOP2B (Hs00172259_m1) were obtained from Applied Biosystems.

Western blot

The whole-cell lysate was prepared with 1% SDS plus 10 mM Tris (pH 7.5) buffer and western blot was performed on 7.5% SDS–PAGE gel as described previously. The primary mouse MAB anti-TOP2A (Enzo Life Sciences; ADI-KAM-CC21) was used at 5 μg/ml dilution and anti-GAPDH (sc-32233; Santa Cruz Biotechnology, Inc.) was used at 1:3000 dilution.

TOP2A inhibitors

A quantitative proliferation assay of drugs that inhibit TOP2A was performed in the NCI-H295R ACC cell line using the CellTiter-Glo Luminescent Cell Viability Assay. The drugs were serially diluted 1:2.24 in DMSO (Thermo Fisher Scientific, Waltham, MA, USA) in 384-well plates. The stock concentrations of the test compounds ranged...
from 10 mM to 0.13 μM. Fourteen TOP2A inhibitors were tested: aclarubicin, idarubicin, teniposide, daunorubicin, doxorubicin, mitoxantrone, amsacrine, topotecan, pirarubicin, rubitecan, etoposide, irinotecan, sarafoxacin, and gatifloxacin. We further validated the antiproliferative effect of aclarubicin (InterBioscreen Ltd., Moscow, Russia) in monolayer and three-dimensional multicellular aggregate (MCA) culture of NCI-H295R and SW13 cell lines.

**Cell proliferation**

Cells were seeded at a concentration of 5000 cells (NCI-H295R) and 2000 cells (SW13) per 150 μl culture medium in a 96-well plate in six replicates. Cells were treated with different drug concentrations based on their half-maximal inhibitory concentration (IC_{50}) obtained from our drug screening using six replicates. The CyQUANT assay kit (Invitrogen) was used to evaluate cell number according to the manufacturer’s instructions. The CyQUANT assay kit (Invitrogen) was used to evaluate cell number according to the manufacturer’s instructions.

**Flow cytometry**

Cells were transfected with TOP2A siRNA and negative control, and after 72 h, cells were harvested, washed with 1× PBS and ethanol-fixed overnight at 4 °C, and resuspended in 1× PBS. Cells were treated with DNase-free RNase (100 μg/ml) for 20 min at 37 °C. The cells were stained with propidium iodide at a concentration of 50 μg/ml and samples were stored at 4 °C. Flow cytometry analysis was performed on a Becton Dickinson FACScan (Franklin Lakes, NJ, USA). Data files were generated for 20,000 events (cells) using CellQuest software (Franklin Lakes, NJ, USA). The fraction of the total cell population present in each of the G1, S, and G2/M cell cycle phases was obtained using ModFit LT software (Verity Software House, Inc. Topsham, ME, USA).

**Apoptosis**

Caspase-Glo 3/7 assay (Promega) was used to measure caspase activity. After 72 h of transfection, 100 μl culture medium was removed from each well, and caspase 3/7 activity was determined using the Caspase-Glo 3/7 assay kit (Promega) according to manufacturer’s instruction.

**Three-dimensional MCAs**

We used a three-dimensional MCA model to mimic an *in vivo* solid tumor and test the anticancer activity of TOP2A inhibitors. A total of 1×10^5 NCI-H295R cells/well and 6×10^4 SW13 cells/well (in 0.5 ml) were plated in 24-well ultra-low attachment plates (Corning Costar, Corning, NY, USA) to generate MCAs. The plates were cultured at 37 °C in 5% CO_2 for 1 week, and the medium was changed every 3 days. After 1 week of culture for NCI-H295R cells and 3 weeks of culture for SW13 cells, distinct MCAs were formed. At these time points, the MCAs were photographed and treated with different concentrations (0.05–1 μM) of aclarubicin or vehicle (DMSO). The MCAs were treated twice a week for 3 weeks and photographed. The quantitation of spheroid activity was done using Image J software (Bethesda, MD, USA) by marking the entire area for each spheroid and calculating the pixel numbers. The quantitation of MCAs (area measurement) was done by Image J software (NIH). All experiments were repeated at least three times.

**Cell invasion assay**

Cell invasion assay was performed as described previously (Jain et al. 2012). Invaded cells were fixed, stained with Diff Quik Stain (Dade Behring, Newark, DE, USA), and counted under a light microscope in four separate fields. The experiments were repeated three times.

**Statistical analyses**

Data are presented as mean ± S.D. or S.E.M. Student’s t-test, two-tailed, was used to compare differences between groups. Kruskal–Wallis, a nonparametric test, was used for comparison of three or more groups. Statistical analysis was done using StatView 5.0 (SAS Institute, Cary, NC, USA) and SPSS v 16.0 (SPSS, Inc.) statistical software. A P value <0.05 was considered as significant.

For the quantitative proliferation assay of the TOP2A inhibitors, titration-response data for each sample were plotted and modeled by a four-parameter logistic fit to determine compound activity. Curve-fits were then classified by criteria described previously (Inglese et al. 2006). Classes 1.1 and 1.2 were full curves containing upper and lower asymptotes with efficacy ≥80 and <80% respectively. Classes 2.1 and 2.2 were incomplete curves having only one asymptote with efficacy ≥80 and <80% respectively. Class 3 curves showed activity at only the highest concentration or were poorly fit. Class 4 curves were inactive having a curve-fit of insufficient efficacy or lacking a fit altogether. Only TOP2A inhibitors with class −1.1, −1.2, and −2.1 curves were considered as high-confidence active compounds.
Results

TOP2A is overexpressed in ACC

TOP2A mRNA expression was significantly higher in ACC than in normal adrenocortical tissue and benign adrenocortical tumors (P<0.008, Fig. 1a). TOP2A mRNA expression was also high in both ACC cell lines and the expression of other topoisomerases such as TOP1 and TOP2B was similar in the two cell lines (Fig. 1b). TOP2A protein expression was also higher in ACC than in benign adrenocortical tumor and normal adrenocortical tissue (Fig. 1c).

Effect of TOP2A knockdown on cellular proliferation, cell cycle, and apoptosis in ACC cell lines

Given the high expression of TOP2A in ACC, we next determined whether TOP2A regulates cell proliferation in ACC cells using siRNA to knockdown TOP2A expression in NCI-H295R and SW13 cells (Fig. 2a). In NCI-H295R, cell proliferation decreased by as much as 30% compared with the negative control (P<0.05, Fig. 2b). In SW13 cells, cellular proliferation was decreased modestly with siRNA knockdown of TOP2A compared with the negative control (P<0.05, Fig. 2c). TOP2A knockdown did not have a significant effect on cell cycle in both cell lines (data not shown) but showed increased apoptosis with TOP2A knockdown compared with control in H295R cells (P<0.05, Fig. 2d).

TOP2A knockdown decreases cellular invasion and soft agar anchorage-independent growth in ACC cells

We were interested in determining whether TOP2A regulates hallmarks of malignant cell phenotype, cellular invasion, and anchorage-independent growth because this gene has been suggested to be only a marker of proliferation and not necessarily mediate malignant cell phenotype. Cellular invasion decreased by 57–71% with TOP2A knockdown compared with negative control in both ACC cell lines (P<0.05, Fig. 2e). We observed
Figure 2
Effect of TOP2A knockdown on ACC cell proliferation, invasion, and colony formation. (a) siRNA knockdown of TOP2A protein expression in ACC cell lines. Total cell lysate was extracted after 7 days of knockdown from siRNA (si#1 and si#3) and negative control (NC) groups in NCI-H295R and SW13 ACC cells and TOP2A protein expression was determined by western blot. (b and c) Cell proliferation in ACC cell lines. The number of NCI-H295R (b) and SW13 (c) cells for TOP2A siRNA (si#1 and #3)-treated and NC-treated groups is shown at 24, 72, 120, and 168 h after transfection. Mean ± S.E.M. **(P < 0.005; relative to NC). Knockdown of TOP2A in ACC cell lines (d) increased apoptosis (NCI-H295R), (e) reduced invasion, and (f) reduced soft agar anchorage-independent growth. siRNA indicates si#3 and NC (negative control). Mean ± S.E.M. * (P < 0.05; relative to NC).
significantly fewer and smaller colonies with TOP2A knockdown ($P=0.006$, Fig. 2f).

**ACC cell lines are sensitive to TOP2A inhibitors**

Given that TOP2A was highly expressed in ACC samples, we evaluated its potential as a therapeutic target for ACC *in vitro*. Eleven of 14 TOP2A inhibitors had an antiproliferative effect in the NCI-H295R ACC cell line, five of the TOP2A inhibitors had a higher antiproliferative activity than mitotane, and aclarubicin was the agent with the highest activity (Fig. 3). The antiproliferative effect of aclarubicin was further validated in monolayer culture of both NCI-H295R and SW13 cell lines at concentrations ranging from 0.05 to 1 μM (Fig. 4a and b). To further confirm the cytotoxic effect of aclarubicin in a three-dimensional model that better mimics solid tumors, it was also administered to NCI-H295R and SW13 MCAs. We observed a significant decrease in the size of MCAs after aclarubicin treatment in both NCI-H295R (Fig. 4c and d) and SW13 cell lines (Fig. 4e and f) at 0.05–0.1 μM ($P<0.05$), which is below the achievable serum concentration of aclarubicin in humans (0.34 μM). However, in NCI-H295R cells, 0.05 μM aclarubicin did not show as dramatic a decrease in MCAs as the higher doses.

**Discussion**

There is a significant need for the development of effective treatment for patients with locally advanced and metastatic ACC. In this study, we analyzed TOP2A expression and function in ACC. We found that TOP2A was overexpressed in ACC and regulates cellular proliferation and invasion. As a consequence of this finding, we screened the antiproliferative activity of TOP2A inhibitors in ACC cell lines and found that most of these agents showed a significant antiproliferative activity and aclarubicin had the most potent anticancer activity.

The function of TOP2A is not well characterized. TOP2A was highly expressed in NCI-H295R and SW13 cell lines. We used gene knockdown strategy to effectively silence its expression. Using this strategy, we observed a modest decrease in cellular proliferation but a dramatic inhibitory effect on anchorage-independent growth and invasion. This is the first study to demonstrate that TOP2A regulates cellular invasion in ACC cells. Our findings are consistent with the association of TOP2A overexpression with the invasiveness of cancers such as liposarcoma and hepatocellular carcinoma (Wong *et al*. 2009, Gobble *et al*. 2011).

Targeting TOP2A with anthracycline drugs is an appealing strategy for the development of effective cancer treatment.
Figure 4
Effect of aclarubicin on NCI-H295R and SW13 cell proliferation and multicellular aggregates (MCA). (a and b) Cell proliferation assay of NCI-H295R and SW13 cell lines 0.05, 0.1, 1.0 μM aclarubicin, and vehicle controls. X-axis indicates days of treatment, and Y-axis indicates the cell number. **P value < 0.005 for comparison of drug treatment vs vehicle control. (c, d, e, and f) Effect of aclarubicin on NCI-H295R (c and d) and SW13 (e and f) MCA. MCAs were treated with 0.05–0.1 μM concentration of aclarubicin and vehicle controls in triplicates. Representative images are shown at 25× magnification. Y-axis indicates total area occupied by spheroids within an image. *P value < 0.05 for the comparison of drug treatment vs vehicle (d and f).
therapy. Aclarubicin is an anthracycline agent that is a strong DNA intercalating agent that prevents the binding of TOP2 to DNA (Sorensen et al. 1992). Several phase I and phase II clinical trials have been performed to evaluate the safety, tolerability, and efficacy of this agent (Jin et al. 2006, Wang et al. 2011). As TOP2A is overexpressed in several malignancies and can be targeted, we studied its expression in ACC and found it to be highly overexpressed in ACCs. We thus hypothesized that TOP2A may be an excellent therapeutic target for ACC. Indeed, we found that 11 of 14 TOP2A inhibitors tested had a significant antiproliferative effect in NCI-H295R ACC cells. Given that aclarubicin exhibited the most potent activity, we validated this finding in monolayer cultures of NCI-H295R and SW13 cell lines. Although monolayer cell cultures can provide cell-specific response to drugs, this model lacks the important features of a three-dimensional solid tumor observed in vivo, such as the hypoxic area of the tumor center, regional differences of tumor growth and cell cycling, as well as poor delivery of drugs into deeper tumor tissue layers. Thus, we used the MCA assay to confirm the anticancer activity of aclarubicin. The effect of aclarubicin was more dramatic in the SW13 cell line than in the NCI-H295R cell line. The difference in sensitivity is not likely due to only the expression levels of TOP2A or other topoisomerases (TOP1 and TOP2B), as the expression levels were similar in both cell lines. Several additional mechanisms may be responsible for the differential sensitivity to aclarubicin we observed in the two cell lines studied. The growth rate of a cell line (SW13 has a shorter doubling time) affects sensitivity to TOP2 inhibitors as demonstrated by Gieseler et al. (1999) who observed higher sensitivity to TOP2 inhibitors in faster growing leukemic cell line (HL-60) compared with KG-1 cells, with slow growth rate. Also, the relative amount of functionally bound topoisomerase to DNA may affect sensitivity to TOP2 inhibitors. Cells with higher fraction (17.53%) of bound topoisomerase to DNA (HL-60) are more sensitive to TOP2 inhibitors than KG-1 cells with fewer fractions (<1%) (Gieseler et al. 1999). Aclarubicin is also an inhibitor of RNA synthesis and p53 and c-myc and this may also mediate differential sensitivity of cancer cells to aclarubicin (Schaefer et al. 1991, Fornari et al. 1996, Morceau et al. 1996, Han et al. 1997). Lastly, Barretina et al. (2012) also recently showed that the unique genetic and genomic background of cancer cell lines cause differential sensitivity or efficacy of anticancer compounds.

Currently, mitotane is the only therapy approved by the US Food and Drug Administration for advanced or metastatic ACC, but it has poor efficacy and a narrow therapeutic window (Luton et al. 1990, Berruti et al. 2005, Terzolo et al. 2007a,b). Compared with mitotane, we found that aclarubicin, idarubicin, teniposide, daunorubicin, and doxorubicin had a lower IC₅₀ and higher efficacy in our screening of the TOP2A inhibitors in the NCI-H295R cell line. Aclarubicin is approved as a second-line therapy for acute myelocytic leukemia in those with refractory disease (Karanes et al. 1983, Hansen et al. 1991, Jeannesson et al. 1997, Kern et al. 1998, Suzushima et al. 2009). Our findings suggest that aclarubicin may be an effective therapeutic alternative in patients with advanced ACC. In a recent randomized control trial comparing mitotane plus combination of etoposide, doxorubicin, and cisplatin (EDP) or streptozocin in patients with unresectable ACC, a higher response rate for the EDP combination regimen was reported (Fassnacht et al. 2012). Given the higher activity we observed for aclarubicin than doxorubicin in ACC cells, future trials should consider aclarubicin in the combination regimen in place of doxorubicin or in combination with only mitotane to determine whether a better response rate may be observed in patients with unresectable ACC.

In summary, this study demonstrates that TOP2A is highly overexpressed in ACC and regulates cellular proliferation, invasion, and anchorage-independent growth in ACC cells. Furthermore, most of the TOP2A inhibitors screened had good antiproliferative activity in ACC cells. Of these, aclarubicin should be further evaluated as a potential therapeutic alternative for patients with locally advanced or metastatic ACC.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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