

Original Article

RNAi-mediated knockdown of α -enolase increases the sensitivity of tumor cells to antitubulin chemotherapeutics

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Abstract: The over-expression of α -enolase was demonstrated in several cancers, including lung, brain, breast, colon and prostate. In this report, we investigated the effects of α -enolase knockdown on the sensitivity of cancer cells to chemotherapeutic drugs. RNAi-mediated knockdown of α -enolase in A549 and H460 lung, MCF7 breast and CaOV3 ovarian cancer cells caused a significant increase in the sensitivity of these cells to antitubulin chemotherapeutics (e.g., vincristine and taxol), but not to doxorubicin, etoposide or cisplatin. This is the first demonstration showing the effects of α -enolase expression on the sensitivity of tumor cells to clinically relevant chemotherapeutics.

Keywords: α -enolase, knockdown, chemotherapeutic drugs, antitubulin chemotherapeutics, sensitivity, cancer, RNAi, A549, H460 lung, MCF7 breast, CaOV3

Introduction

Enolase is an abundantly expressed glycolytic enzyme that catalyzes the dehydration of 2-phospho-D-glycerate into phosphoenolpyruvate, the second ATP production step in the glycolytic pathway [1]. Three different enolase isoenzymes are found in vertebrates: α -enolase is expressed in most tissues, β -enolase is muscle-specific, and γ -enolase is found in tissues of the nervous system [2]. The three enolase isoforms are encoded by distinct genes, but their amino acid sequences show remarkable phylogenetic conservation across species [3]. High level α -enolase expression has been demonstrated in the plasma of patients with lung, breast and prostate carcinomas [4]. The neural-specific enolase (γ -enolase) has been widely used as a diagnostic marker for neuroendocrine tumors and small cell lung carcinomas [5]. Moreover, a strong correlation was observed between serum γ -enolase levels and clinical response to chemotherapy [6]. Over the past decade several other non-glycolytic functions have been ascribed to this enzyme [7], including a structural function

whereby α -enolase or τ -crystallin, is one of the most abundant structural proteins in vertebrate lens [8]. Furthermore, α -enolase is present on the surface of a variety of hematopoietic cells [9], as well as neuronal [10] and endothelial cells [11]. Cell surface α -enolase functions as a plasminogen receptor [7]. Enolase and several glycolytic enzymes also interact with microtubules and F-actin filaments [12, 13]. Enolase was found to localize to centromeres and microtubules in HeLa cells [14]. Thus we hypothesized that enolase-tubulin interactions could affect the sensitivity of tumor cells to anti-mitotic chemotherapeutic drugs. In this report, we examined the effect of RNAi-mediated knockdown of α -enolase on the sensitivity of tumor cells to anti-cancer drugs. Our results show that knockdown of α -enolase expression in different tumor cell lines caused a dramatic increase in their sensitivity to microtubule targeting drugs (e.g., taxanes and vincristine). The results of this study suggest that α -enolase expression levels can affect the sensitivity of tumor cell lines to anti-tubulin drugs, possibly due to α -enolase-tubulin interactions.

Materials and methods

Tissue cell culture

All cell culture materials and reagents were obtained from Gibco Life Technologies (Burlington, Ont., Canada), with the exception of the drugs that were purchased from Sigma Chemical (St. Louis, MO, USA). Cells were cultured in α MEM medium (MCF-7 cells), in RPMI-1640 medium (H460 cells), in DMEM high glucose medium (CaOV3 cells), or in Ham's F12 medium A549 cells. All growth media were supplemented with 10% fetal bovine serum. The cells were grown in the absence of antibiotics at 37 °C in a humid atmosphere of 5% CO₂ and 95% air. All cell lines were examined for and determined to be free of mycoplasma contamination using a PCR-based mycoplasma detection kit according to manufacturer's instructions (Stratagene Inc., San Diego, CA, USA).

RNA Interference

Predesigned siRNA duplexes targeting the human α -enolase mRNA were purchased from Invitrogen (e.g., sense strand 5'-CUCAAAGGCUG UUGAGCACAUCAAU-3' targeting nucleotides 337-352 of the α -enolase mRNA from RefSeq NM_001428). As a negative control, the scrambled sequence 5'-CCAGGGUCCUAAUCGGAUUU GCUA-3' without significant homology to any human gene was also designed and obtained from Invitrogen. Cells were transfected with scrambled or α -enolase-specific siRNA as previously described [15]. Transfection efficiencies were typically evaluated 24-48 hrs post transfection using Cy3 labeled GL2 siRNA duplex and efficiencies of transfection were routinely greater than 95%. For a typical siRNA transfection, 1 nmole of the annealed siRNA duplex was mixed with 1.4 ml of Opti-MEM reagent (Invitrogen), and separately 85 μ l of Oligofectamine reagent was mixed with 600 μ l of Opti-MEM. The two solutions were combined and mixed gently by inversion and incubated for 20 min at room temperature. The resulting solution was added drop-wise to 40-50% confluent cells in a 10-cm dish.

Cell extraction and Western blotting

Cells were rinsed twice with phosphate buffer saline (PBS), and harvested by trypsinization. The cell pellets were lysed in 50-150 μ l of lysis

buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate), containing protease inhibitors (1 μ g/ml pepstatin, 1 μ g/ml leupeptin; 1 μ g/ml benzamidin; 0.2 mM PMSF). The cell lysates were then centrifuged at 14,000xg for 10 min at 4°C and protein concentrations of the supernatants were determined by the DC Protein assay (BioRad). For Western blot analysis, total cell lysates (100 μ g/well) from mock, scrambled siRNA, or α -enolase-siRNA transfected cells were resolved on 10% SDS-PAGE gels [16] and transferred onto nitrocellulose membranes (Hybond, Amersham Pharmacia Biotech) for 1 hour [17]. Membranes were incubated in 5% non-fat milk in PBS overnight at 4 °C, prior to the addition of the primary antibodies (e.g., rabbit polyclonal antibody against human α -enolase from Santa Cruz Biotechnology and anti-GAPDH from Novus Biologicals) for 2 hrs incubation, followed by an 1-hr incubation for HRP-coupled secondary antibodies (e.g., goat anti-rabbit and goat anti-mouse antibodies). The signal was detected by the Supersignal Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA). Quantification of α -enolase expression was done by densitometric analysis using Scion Image software (Scion Corp.) with normalization to GAPDH expression.

Cytotoxicity assay

siRNA-transfected cells were harvested 48 hrs post-transfection by trypsinization and were seeded in triplicate into 96-well plates at 5x10³ cells/well. Cells were incubated for an additional 16-24 hrs prior to drug exposure. Cells were incubated in increasing concentrations of drugs (e.g., doxorubicin, vincristine, mitoxantrone, taxol, vinblastine, etoposide, docetaxel, or cisplatin) for an additional 72 hrs prior to assay development with the addition of 25 μ l/well of MTT dye (5 mg/ml). The cells were further incubated at 37 °C for 4 hrs, and the assay was terminated with the addition of 10% Triton X-100 in 0.01 N HCl. The absorption at 570 nm was used to assess the relative cell viability, and the averages of triplicate wells from three independent experiments were plotted using the Prism software (GraphPad).

Results and discussion

To investigate the potential role of α -enolase on the sensitivity of tumor cells to anti-cancer drugs, we employed RNAi to silence its expres-

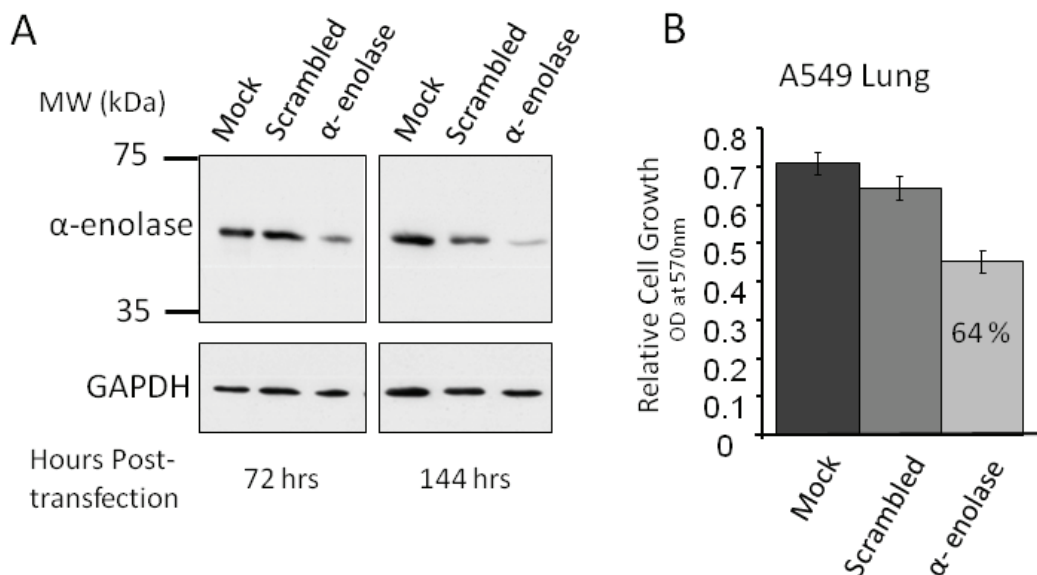


Figure 1. Knockdown of α -enolase expression in A549 lung tumor cells by RNAi. A549 cells were mock transfected or transfected with 1 nmole of α -enolase-specific or scrambled siRNA duplexes. The effect of α -enolase siRNA on the protein expression in A549 cells was assessed by Western blotting 72 hrs and 144 hrs post-transfection (1A). Total cell extracts from mock, scrambled or α -enolase-specific siRNA were evaluated using a rabbit polyclonal antibody against α -enolase (Santa Cruz Biotechnology). The level of α -enolase knockdown in A549 cells was compared to the same cells transfected with scrambled siRNA and the levels of GAPDH as a house-keeping internal control protein. Figure 1B shows the growth of A549 cells transfected with α -enolase siRNA versus mock or scrambled siRNA-transfected A549 cells in the absence of drugs.

sion in several tumor cell lines. Pre-designed siRNA duplexes targeting human α -enolase or control scrambled siRNA were transfected into A549 lung tumor cells. **Figure 1A** shows Western blotting of protein extracts from A549 cells transferred to nitrocellulose membrane and probed with antibodies to human α -enolase and GAPDH. The results in **Figure 1** show that transfection of α -enolase specific siRNA, but not mock or scrambled siRNA, caused 77-90% knockdown of α -enolase in A549 cells relative to GAPDH expression. Transfection of α -enolase-specific siRNA in other tumor cell lines (e.g., H460 lung, MCF7 breast and CaOV3 ovarian cells) caused similar knockdown of α -enolase relative to GAPDH expression (data not shown). α -enolase knockdown in A549 cells caused a 36% decrease in cell growth relative to mock transfected or cells transfected with scrambled siRNA (**Figure 1B**). The decrease in cell growth following α -enolase knockdown indicated the importance of the glycolytic function of the protein and increased aerobic glycolysis in tumor cells [18]. Hence a decrease in the expression level of one or more glycolytic enzymes will likely

affect the ATP levels and consequently the growth of A549 cells. In fact it is somewhat surprising, given the reliance of tumor cells on aerobic glycolysis, that knockdown of α -enolase expression by 77-90% in A549 (as quantified by scanning of α -enolase bands and normalized to GAPDH expression in **Figure 1A**) did not cause a greater decrease in cell growth. One possibility may be that reduction in aerobic glycolysis in tumor cells shifts their metabolic energy reliance to oxidative phosphorylation [18].

To evaluate the effects of α -enolase knockdown on the sensitivity of cells to cytotoxic agents, proliferation assays were performed on cells transfected with α -enolase-specific siRNA at 72 hrs post-transfection and an MTT assay was performed at 144 hrs. **Figure 2** shows the viability of A549 cells transfected with α -enolase or scrambled siRNA after 72 hr incubation with increasing concentrations of doxorubicin, vincristine, mitoxantrone, taxol, vinblastine, etoposide, docetaxel, or cisplatin. Knockdown of α -enolase caused a marked increase in the sensitivity of A549 cells to antitubulin drugs (e.g.,

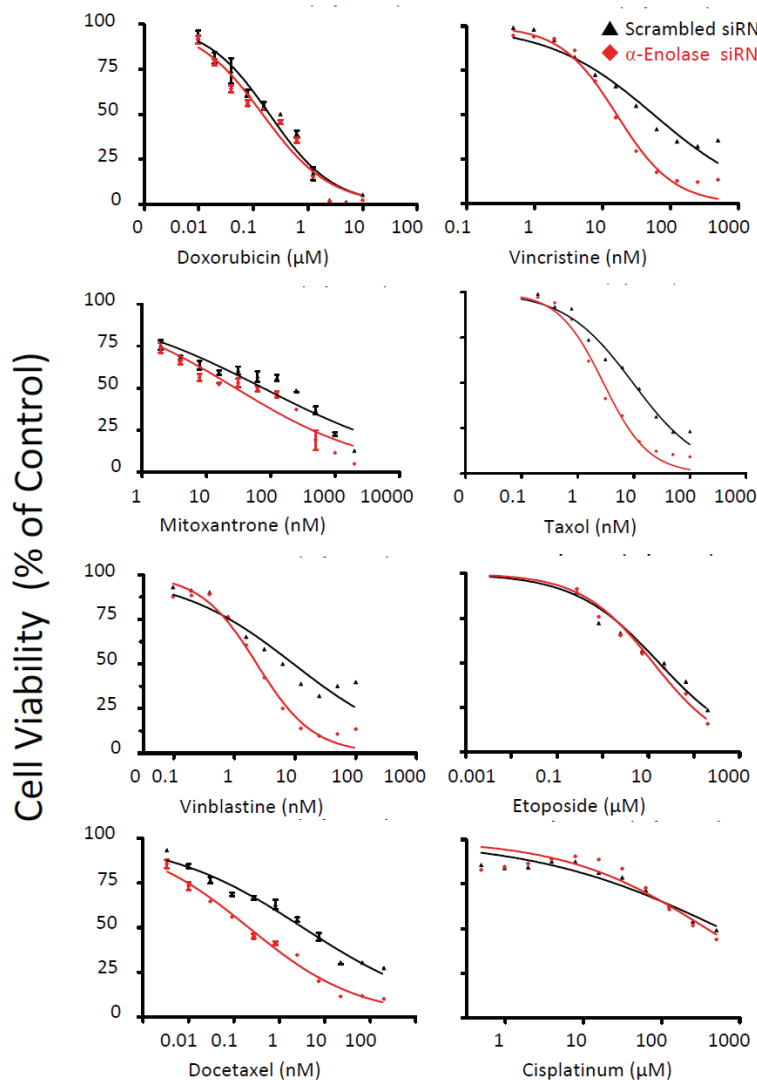


Figure 2. Effects of cytotoxic drugs on the growth of A549 cells transfected with α -enolase or control scrambled siRNA. Two days post transfection with α -enolase siRNA duplexes, cells were seeded in triplicate in 96-well plates and further incubated for an additional 16 hours prior to their incubation with increasing concentrations of cytotoxic drugs. Results are expressed as percent viability compared to cells treated with scrambled siRNA. The averages of triplicate wells were plotted and the IC_{50} values for each drug were derived from these graphs using the Prism software. Three independent experiments were performed for each cell line and drug type.

vincristine, vinblastine, taxol and docetaxel), while the sensitivity of A549 cells to doxorubicin, etoposide or cisplatin was not significantly affected (**Figure 2**). A small, but significant, increase in the sensitivity of these cells to mitoxantrone was observed. Given the results in **Figure 2**, it was of interest to test the effects of two clinically useful antitubulin chemotherapeutics in other tumor cell lines. **Figure 3** shows the effects of α -enolase knockdown on the sensitivity of H460 lung, MCF7 breast and CaOV3 ovarian cancer cells to vincristine and taxol. These results show a consistent increase in the sensitivity of all tumor cell lines to vincristine and taxol, replicating the increased drug sensitivity of A549 cells. **Table 1** summarizes the fold decreases in IC_{50} for each tumor line tested for

vincristine and docetaxel, respectively. Collectively, the above results suggest that α -enolase may possess pro-survival functions and changes in its expression could affect the sensitivity of tumor cells to certain anti-cancer drugs. The latter possibility is supported by an earlier study whereby α -enolase was reported to be an endothelial hypoxic stress protein [19]. Thus, up-regulation of α -enolase in response to hypoxia is believed to promote cell survival under hypoxic stress, perhaps through increased ATP production

by enhancing glycolysis [20]. Moreover, the induction of α -enolase expression was shown to be mediated by HIF-1 α through hypoxia response elements in its promoter [21]. Interestingly, VEGF is also regulated in a similar manner by hypoxia and HIF-1 α [22]. VEGF is a pro-survival growth factor in vascular endothelial cells [23] and has been reported to promote drug resistance of endothelial cells to microtubule targeting agents [24]. Thus, a link between VEGF signaling and α -enolase suggests a role for the latter as pro-survival protein which is consistent with our findings in this study. Neovascularization and increased glycolysis are two common characteristics of solid tumors. Furthermore, recent chemotherapeutic regimens have promoted the use of anti-angiogenic

α -enolase knockdown increase sensitivity to antitubulin drugs

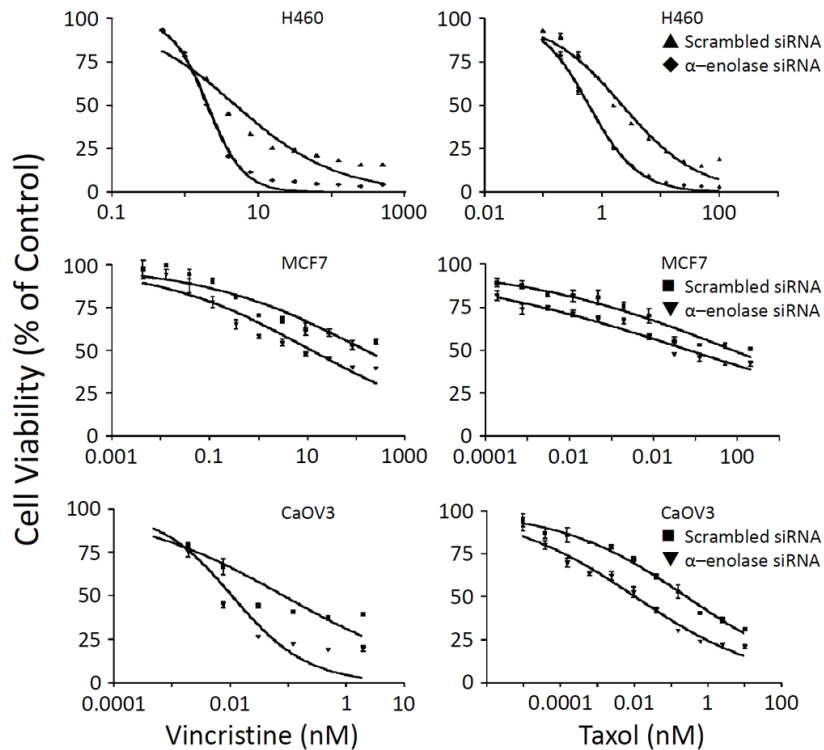


Figure 3. Effect of α -enolase knockdown on the sensitivity of tumor cells to vincristine and taxol. Two days post transfection with α -enolase or control scrambled siRNA, H460 lung, MCF7 breast, CaOV3 ovarian cancer cells were seeded in triplicate in 96-well plates and further incubated for 16 hours prior to the addition of increasing concentrations of vincristine or taxol.

Table 1. Summary of the effects of α -enolase knockdown on the sensitivity of tumor cells to antitubulin chemotherapeutics

Cell Line	Vincristine	Taxol
	Fold decreases in IC ₅₀	
A549 Lung	4.2	2
H460 Lung	2.5	3.7
MCF-7 Breast	12.9	10.9
CaOV3 Ovarian	7.8	24.6

Note: The results show the fold decrease in IC₅₀ values of the tumor cells transfected with α -enolase-siRNA relative to CTL siRNA.

drugs such as VEGF inhibitors in combination with low dose chemotherapy [25]. Our results suggest a possible molecular basis for this combination treatment through down-regulation of α -enolase expression. The observed increase in sensitivity of tumor cells to antitubulin chemot-

herapeutics may be due to previously demonstrated interactions between α -enolase and tubulin or microtubule structures [26, 27]. Thus, we speculate that α -enolase binding to microtubules could compete with taxol and vincristine binding sites as reported for two other glycolytic enzymes, phosphofructokinase and pyruvate kinase [28]. Moreover, α -enolase has been recently reported to co-pellet with taxol-stabilized microtubules [26], and as such our results are also consistent with a role for α -enolase in modulating microtubule network. In summary, the findings in this report show that RNAi-mediated knockdown of α -enolase in several tumor cell lines increases their sensitivity to antitubulin drugs and could have implications in cancer treatment.

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Abbreviations: Inhibitory RNA, RNAi or siRNA; Drug concentration that inhibits the growth of cells by 50%, IC₅₀; Vascular endothelial growth factor, VEGF; Glyceraldehyde 3-phosphate dehydrogenase, GAPDH; Hypoxia-inducible factor-1 alpha; HIF-1 α ; (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT.

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