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An RNA-directed nucleoside anti-metabolite, 1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl)cytosine (ECyd), elicits antitumor effect via *TP53*-induced Glycolysis and Apoptosis Regulator (TIGAR) downregulation

Vivian Wai Yan Lui [a,1], Cecilia Pik Yuk Lau [a,1], Crystal Sao Fong Cheung [a], Kakiu Ho [a], Margaret Heung Ling Ng [b], Suk Hang Cheng [b], Bo Hong [a], Sai-Wah Tsao [c], Chi Man Tsang [c], Kenny Ieng Kit Lei [a], Yasundo Yamasaki [d], Akira Mita [d], Anthony T.C. Chan [a,*]

[a] *Cancer Drug Testing Unit, State Key Laboratory in Oncology in Southern China, Sir Y.K. Pao Center for Cancer, Department of Clinical Oncology, Hong Kong Cancer Institute and Li Ka Shing Institute for Health Sciences, Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China*
[b] *Department of Anatomical and Cellular Pathology, Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China*
[c] *Department of Anatomy, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong, China*
[d] *Taiho Pharmaceutical Co., Ltd., Tokyo, Japan*

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ABSTRACT

1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl)cytosine (ECyd) is a ribose-modified nucleoside analog of cytidine with potent anticancer activity in several cancers. The main antitumor mechanism of this promising RNA-directed nucleoside anti-metabolite is efficient blockade of RNA synthesis in cancer cells. Here, we examined the therapeutic potential of this RNA-directed anti-metabolite in *in vitro* models of nasopharyngeal cancer (NPC). In a panel of 6 NPC cell lines, ECyd effectively inhibited cellular proliferation at nM concentrations (IC_{50}:~13–44 nM). Moreover, cisplatin-resistant NPC cells were highly sensitive to ECyd (at nM concentration). The ECyd-mediated growth inhibition was associated with G_2/M cell cycle arrest, PARP cleavage (a hallmark of apoptosis) and Bcl-2 downregulation, indicating induction of apoptosis by ECyd in NPC cells. Unexpectedly, ECyd-induced significant downregulation of TIGAR, a newly described dual regulator of apoptosis and glycolysis. More importantly, this novel action of ECyd on TIGAR was accompanied by marked depletion of NADPH, the major reducing power critically required for cell proliferation and survival. We hypothesized that ECyd-induced TIGAR downregulation was crucially involved in the antitumor activity of ECyd. Indeed, overexpression of TIGAR was able to rescue NPC cells from ECyd-induced growth inhibition, demonstrating a novel mechanistic action of ECyd on TIGAR. We demonstrated for the first time that an RNA-directed nucleoside analog, ECyd, exerts its antitumor activity via downregulation of a novel regulator of apoptosis, TIGAR. Moreover, ECyd may represent a novel therapy for NPC.

1. Introduction

RNA targeting has been shown to be effective for cancer therapy as tumor cells rely on active gene transcription for sustained tumor growth, survival, anti-apoptosis and angiogenesis. Blockade of RNA synthesis and induction of RNA degradation are two major RNA targeting strategies. Various RNA-directed therapies, including gene therapy approaches (e.g. ribozyme, RNA interference, antisense therapy, etc) [1,2], RNA polymerase inhibitors (e.g. DRB, CYC202, flavopiridol) [3–6], as well as RNA-directed nucleoside analogs (e.g. 8-chloro-adenosine, ARC, EUrd, etc) [7–9] have demonstrated promising antitumor activities *in vitro* and *in vivo* [10–12]. It is known that tumor cells constantly over-produce or overexpress large amount of important transcripts (thus proteins) with high turn-over rates for their survival, thus hampering the rapid rate of RNA synthesis would theoretically be more effective than approaches that enhance degradation of such a large amount of over-produced transcripts in tumor cells. By blocking active transcription, short-lived survival factors are effectively depleted or reduced, which

Abbreviations: ECyd, 1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl)cytosine; NPC, nasopharyngeal carcinoma; LMP1, latent membrane protein 1; TIGAR, *TP53*-induced glycolysis and apoptosis regulator; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; ARC, 4-amino-6-hydrazino-7-beta-D-ribofuranosyl-7H-pyrrolo(2,3-d)-pyrimidine-5-carboxamide; EUrd, 1-(3-C-ethynyl-beta-D-ribopentofuranosyl)uracil.

* Corresponding author at: Department of Clinical Oncology, Prince of Wales Hospital, Hong Kong. Tel.: +852 2632 2099; fax: +852 2648 8842.
E-mail address: anthony@clo.cuhk.edu.hk (Anthony T.C. Chan).

[1] Co-first authorship.

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contributes to tumor cell death. Previous studies demonstrated that important survival or apoptosis factors such as c-Met, cyclin E, Bcl-2, Mcl-1, XIAP were downregulated by transcriptional blockers in cancer cells, which is believed to contribute to subsequent cell death [5,8,9,13].

ECyd, 1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl)cytosine, is a ribose sugar-modified cytosine analog (or ribonucleoside analog) with potent antitumor activity in several human cancer models [12,14–16]. Promising results from preclinical antitumor efficacy and pharmacokinetic studies of this agent [17] have recently led to Phase I, and currently Phase II human clinical trials for efficacy evaluation in solid tumors [18]. This cell permeable small molecule drug has been demonstrated to be an effective RNA-targeting agent, inhibiting over 80–90% of RNA synthesis in tumor cells [7,14] and is able to initiate tumor cell death effectively. ECyd is an inactive small molecule pro-drug, when it enters the cells, it is metabolically converted to the active cytotoxic drug, ECyd 5′-triphosphate (ECTP). As ECTP is structurally similar to the intracellular cytidine 5′-triphosphate, CTP (a substrate for RNA synthesis), therefore ECTP competes with CTP for RNA polymerase and results in competitive inhibition of RNA polymerase activity, thus subsequent blockade of RNA synthesis [7,14,19,20].

Uridine–cytidine kinase (UCK) is a metabolic enzyme required for the very first step in a series of bio-conversion processes (addition of mono, bi and triphosphate) of ECyd to the active anti-metabolite, ECTP [7]. It has been shown that tumors harbor high UCK activity than normal tissues, thus allowing more effective bio-conversion of ECyd in the tumors for it to elicit its antitumor activity [7]. Elucidation of the detailed antitumor mechanism of ECyd reveals the involvement of activation of caspase-dependent DNA fragmentation (with subsequent apoptosis induction), activation of RNase L-mediated RNA degradation pathway [19], as well as downregulation of HIF-1α in hypoxic tumor cells [21]. Studies are ongoing to examine if other antitumor mechanisms are involved.

Nasopharyngeal cancer (NPC) is an Epstein-Barr virus (EBV)-associated head and neck cancer prevalent in Asia, with an annual incidence of 15–30/100,000 persons (comparable to that of pancreatic cancer in the US) [22,23]. Disease recurrence and distant metastasis account for major fatality of the disease. Over 60–70% of NPC patients have advanced disease (Stage IIb or IV) at the time of diagnosis. Even with the most aggressive concurrent chemo-radiotherapy, still 30–40% of patients fail with poor survival and disease recurrence. Therefore, more effective therapy is much needed. In this study, we demonstrated that this potent RNA synthesis inhibitor, ECyd, effectively inhibited proliferation of multiple NPC cell lines, as well as cisplatin-resistant NPC cell lines at nM concentration. Growth inhibition of NPC cells was associated with cell cycle arrest in G_2/M phase, PARP cleavage (a hallmark of apoptosis) and downregulation of the anti-apoptotic protein, Bcl-2. Strikingly, ECyd (at nM concentration) significantly reduced the expression of TIGAR (a newly described dual regulator of apoptosis and glycolysis) with marked depletion of intracellular NADPH, the major reducing power required for cell survival and proliferation (TIGAR has been postulated to inhibit apoptosis via regulation of cellular NAPDH levels). We hypothesized that ECyd-induced downregulation of TIGAR may represent a novel antitumor mechanism of this ribose-based nucleoside anti-metabolite. Indeed, overexpression of TIGAR rescued NPC cells from ECyd-induced growth inhibition, demonstrating the mechanistic importance of TIGAR down-modulation by ECyd. Our findings not only demonstrated the therapeutic potential of ECyd in NPC, but also revealed a novel antitumor mechanism of ECyd.

2. Materials and methods

2.1. Reagents

ECyd was obtained from Taiho Pharmaceutical Co. Ltd., Japan. UCK-EIA kit was from Immuno-Biological Laboratories Co. Ltd., Japan. Sources of antibodies used in the study were: anti-cleaved PARP (#9541S) from Cell Signaling Technology, Beverly, MA; anti-p53 (DO-1) (sc-126) and c-kit (SC-168) antibodies from Santa Cruz Biotechnology, Santa Cruz, CA; anti-TIGAR (ab37910) from Abcam, Cambridge, UK; anti-Bcl-2 (124) (M0887) from DakoCytomation, Glostrup, Denmark; anti-β-actin antibody from Calbiochem, Merck, San Diego, CA and secondary antibodies from Invitrogen, Carlsbad, CA. Cisplatin was obtained from Mayne Pharma Pty Ltd., VIC, Australia.

2.2. NPC cell cultures

Human NPC cell lines, CNE-2, HONE-1, HONE-1-EBV, C666-1, HK1, HK1-LMP1 were cultured and maintained as previously described [24,25]. Cisplatin-resistant NPC cell lines, HONE-1-EBV-CisR clones and HK1-LMP1-CisR clones were developed as previously described [26]. Cisplatin-selection medium was removed from the cisplatin-resistant clones 3 days before experiments.

2.3. ECyd treatment

ECyd was prepared as a 10 mM stock in sterile double-distilled H_2O, aliquoted and stored at 4 °C. ECyd was diluted to the desired concentrations with complete RPMI (Thermo Scientific Hyclone, Waltham, MA) and added to the cells for the indicated period of time. Vehicle control (medium only) was included in all experiments.

2.4. Human uridine–cytidine kinase-2 (UCK-2) enzyme immunoassay (EIA)

Total cellular protein was extracted from NPC cells by homogenization. One microgram of total protein was subjected to UCK-EIA according to manufacturer's instruction. In brief, 100 μl (0.01 mg/ml) protein lysate was incubated with the EIA buffer in a 96-well plate at 4 °C overnight. After 7 washings with 0.05% Tween 20 in phosphate buffer, HRP-conjugated anti-human UCK-2 antibody was added into each well and incubated for 30 min at 37 °C, followed by 9 washings with 0.05% Tween 20 in phosphate buffer, and incubation with freshly prepared tetra methyl benzidine (TMB) buffer (100 μl) for 30 min at room temperature. The reaction was stopped by the addition of 100 μl 1N H_2SO_4 (100 μl) and absorbance was measured at 450 nm with a microplate reader (PerkinElmer 1420 Multilabel Counter VICTOR3, Waltham, MA). UCK-2 concentrations in the samples were determined from a standard curve prepared with UCK-2 standard (0–6.25 ng/ml UCK-2). Assay was repeated three independent times, each with duplicate (total $n = 6$).

2.5. Cell viability assay

The effect of ECyd on NPC cell proliferation was assessed by MTT assay as previously described [26]. NPC cells were cultured in 48-well plates (500–5000 cells per well) in complete RPMI. Cells were treated with 0.01 nM, 1 nM, 10 nM, 50 nM, 0.5 μM, 5 μM, and 20 μM of ECyd or vehicle for 24 h, 48 h, or 72 h. Cisplatin-resistant cells were treated with ECyd at 50 nM and 200 nM (about IC_{50} and IC_{75} concentrations at 48 h, respectively). Cells were then subjected to MTT assay. Percentage of growth inhibition was calculated as $(OD_{vehicle} - OD_{treatment})/OD_{vehicle} \times 100\%$, where OD was measured at 570 nm with a microplate reader. The drug

concentration at which 50% of maximum growth inhibition was achieved was determined as the IC_{50} using the GraphPad PRISM software (Intuitive Software for Science, La Jolla, CA). In each experiment, triplicate wells were performed for each drug concentration, and assay was repeated in three independent experiments.

2.6. Cell cycle analysis

Cells were plated in 50 mm^2 Petri dishes (1.2×10^5) and then treated with ECyd for 24 h and 48 h. Cells were collected by trypsinization, fixed with 70% cold ethanol and stored at −20 °C. DNA staining was performed with a solution containing 1 mg/ml RNase A (USB, Cleveland, OH) and 25 μg/ml propidium iodide (Sigma–Aldrich, St. Louis, MO). Analysis was performed using a FACScan flow cytometer while data of cell cycle were processed with CELLQuest software (Becton Dickinson, Franklin Lakes, NJ). All assays were repeated in three independent experiments.

2.7. Western blotting

Cells were treated with ECyd at 50 nM and 500 nM (about IC_{50} and IC_{max} concentrations at 48 h, respectively). Total cellular protein was extracted and 50 μg of the total protein was subjected to SDS-PAGE and immunoblotting as previously described [27]. For detection of apoptosis markers, the expression of cleaved PARP, Bcl-2, p53 and TIGAR was determined. Assay was repeated in three independent experiments.

2.8. Cell death detection ELISA

ECyd-induced cell death was determined using the Cell Death Detection ELISAPLUS kit (Roche Diagnostics, Berlin, Germany), according to manufacturer's instruction. This photometric enzyme immunoassay measures cell death by quantifying the cytoplasmic histone-assoicated-DNA-fragments. In brief, HONE-1, HONE-1-EBV and HK1-LMP1 cells (1.2×10^5 cells/plate) were plated and treated with 50 nM and 500 nM concentration of ECyd or vehicle for 48 h. Tumor cells were then lysed with lysis buffer. Cell lysates were then incubated with the immunoreagent (Biotinized anti-histone and peroxidase conjugated anti-DNA–POD mixture) in a streptavidin coated-plate for 2 h at room temperature, then washed 3 times with incubation buffer, and subsequently incubated with ABTS (2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate for 5 min. Absorbance was measured at 405 nm and 490 nm. Apoptosis induced by the drug was presented as fold change in apoptosis (vs vehicle control) = OD (405–490 nm)$_{ECyd}$/OD (405–490 nm)$_{veh.}$

2.9. NADPH assay

Cellular NADPH level was determined according to manufacturer's instruction (EnzyChrom™ $NADP^+$/NADPH assay kit, Bioassay Systems, Hayward, CA). Briefly, HONE-1, HONE-1-EBV and HK1-LMP1 cells (2×10^5 cells/well) were plated and treated with 500 nM concentration of ECyd or vehicle for 48 h. Cells were lysed and cellular NADPH was extracted using extraction buffer, followed by incubation at 60 °C for 5 min and centrifugation for 5 min at 14,000 rpm. Working reagent containing glucose, MTT, phenazine methosulfate (PMS) and glucose dehydrogenase was added to the supernatant of the samples and standard to determine the NADPH level. NADPH level in the samples was determined from the absorbance of the samples at 570 nm at 30 min (with background measurement at 0 min) with reference to the standard curve. Protein concentration in the samples was determined by protein quantification as mentioned [27]. The NADPH concentration was normalized to total protein and presented as μg/min/mg total protein.

2.10. RT-PCR

Total RNA was extracted from NPC cells using Trizol reagent (Invitrogen, Carlsbad, CA). Prior to RT-PCR, the purified RNA was pre-treated with DNase I (Fermentas Life Sciences, Ontario, Canada). RT-PCR was performed with 0.1 μg of DNase I-treated RNA using Access RT-PCR System (Promega, Madison, WI). Specific primers for RT-PCR of the human *TIGAR* and *p53* transcripts were: *TIGAR* forward primer: 5′-CTCCAGTGATCTCATGAGG-3′ and TIGAR reverse primer: 5′-AGACACTGGCTGCTAATC-3′; *p53* forward primer: 5′-CTGAGGTTGGCTCTGACTGTACCACCATCC-3′ and *p53* reverse primer: 5′-CTCATTCAGCTCTCGGAACATCTCAAGCG-3′ (Invitrogen, Carlsbad, CA). The reaction condition for both transcripts was 45 °C for 45 min, 94 °C for 2 min, followed by 33 cycles of amplification at 94 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s; with a final extension at 72 °C for 7 min. RT-PCR products were analyzed on a 1.2% agarose gel with ethidium bromide and photographed. *GAPDH* was used as the loading control (primer sequences for GADPH were as previously described [28]).

2.11. TIGAR transfection in NPC cells

HONE-1 and HONE-1-EBV cells (1.0×10^4 cells/well and 1.4×10^4 cells/well, respectively) were seeded onto 24-well plates and incubated for 24 h overnight. NPC cells were then transfected with 0.5 μg of TIGAR expression plasmid (OriGene, Rockville, MD) or vector control (pcMV6-XL5) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for an additional 24 h. ECyd (500 nM) or vehicle was added to the transfectants and incubated for additional 24 h (for HONE-1) or 48 h (for HONE-1-EBV), respectively. Effects of TIGAR overexpression on ECyd-induced growth inhibition were assayed by MTT assay.

2.12. Statistical analysis

Statistical analyses were performed using PRISM4 Software (GraphPad, La Jolla, CA) for unpaired *t*-test with Welch Correction. Results were considered as significant with p-value <0.05.

3. Results

3.1. ECyd inhibits NPC cell growth at nanomolar (nM) concentrations

First, we examined the effect of ECyd on NPC cell growth in a panel of 6 NPC cell lines with various differentiation status (HONE-1, HONE-1-EBV and CNE-2 were derived from poorly differentiated NPC; HK1 and HK1-LMP1 were from differentiated NPC; C666-1 was from undifferentiated NPC). As shown in Fig. 1(A), ECyd elicited a dose-dependent growth inhibition in all NPC cell lines with IC_{50} values of 13–44 nM at 48 h (Fig. 1(B)). A time-course study showed that the growth inhibitory effect of ECyd was observed as early as 24 h, with increased efficacy over time. At 72 h, a marked growth inhibition of 94–99% was observed in all NPC cell lines at 500 nM concentration, except in C666-1, 85% growth inhibition was observed (Fig. 1(C)). The nanomolar sensitivity of NPC cells towards ECyd implicates efficient bio-conversion of this pro-drug to the active cytotoxic form, ECTP in NPC cells. Indeed, uridine-cytidine kinase (UCK, a key enzyme involved in the first step of ECyd-ECTP bio-conversion), in particular the UCK-2 isoform, was found to be abundantly expressed in all NPC cell lines (ranging from 113 ng/mg to 608 ng/mg total protein) (Fig. 1(D)), which agrees with the

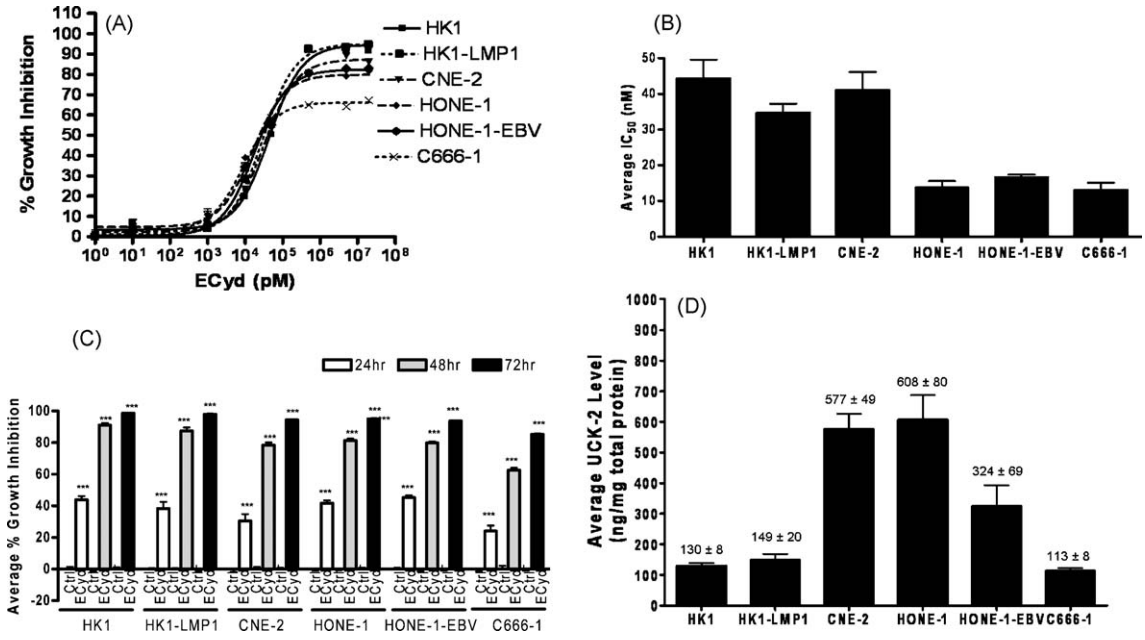

Fig. 1. Dose-dependent inhibition of NPC cell proliferation by ECyd. A panel of 6 human NPC cell lines, HK1, HK1-LMP1, HONE-1, HONE-1-EBV, CNE-2, and C666-1 were treated with ECyd (0–20 μM) for 24 h, 48 h or 72 h, followed by MTT assay. Percent growth inhibition vs respective control culture was shown. (A) Representative dose–response curves of 6 human NPC cell lines at 48 h ($n = 3$). (B) Average IC_{50} values of all NPC cell lines at 48 h (average of 3 independent experiments, total of $n = 9$). (C) Average maximum growth inhibition (%) of all NPC cell lines at 500 nM ECyd vs vehicle control (Veh) at 24 h, 48 h, and 72 h (average of three independent experiments, total of $n = 9$, $^{***}p < 0.0001$). (D) All 6 NPC cell lines expressed abundant and detectable levels of UCK-2. The cellular UCK-2 expression was determined by enzyme immunoassay (EIA). The average levels from 3 independent experiments ($n = 6$) were shown.

previous finding that UCK-2-expressing cancer cells are sensitive to ECyd, while UCK-2-deficient cells are ECyd-resistant [29,30].

3.2. ECyd induces G_2/M arrest and apoptosis in NPC cells

Next, we examined the effect of ECyd on cell cycle progression and apoptosis in NPC cells. The most sensitive EBV-associated NPC cell lines (with the highest maximal killing and lowest IC_{50}) of poorly differentiated and differentiated origins, HONE-1-EBV and HK1-LMP1, were chosen respectively for further study. The parental cell line for HONE-1-EBV, namely HONE-1 was also included for comparison to help defining potential effects of ECyd related to EBV-infection since ECyd has not been studied in other EBV-associated cancers previously. As shown in representative cell cycle histograms (Fig. 2(A)), ECyd (500 nM) induced a significant cell cycle arrest at G_2/M phase at 48 h, with a concomitant reduction in G_0/G_1 phase in all 3 NPC cell lines tested. Cumulative data indicated that there was an average increase of $14 \pm 1\%$, $16 \pm 4\%$ and $25 \pm 3\%$ ($n = 3$) in G_2/M populations induced by ECyd in HONE-1, HONE-1-EBV and HK1-LMP1, respectively. Similar trends were observed in all 3 NPC cell lines as early as 24 h (Supplementary Fig. 1). In addition to cell cycle arrest, ECyd was also able to induce apoptosis as indicated by marked induction of PARP cleavage, and downregulation of the anti-apoptotic protein, Bcl-2 in all 3 NPC cell lines at 48 h (Fig. 2(B)). Quantitative analysis of apoptosis by TUNEL ELISA assay demonstrated that ECyd (vs vehicle) induced a 2-fold ($n = 6$, $^{*}p = 0.028$), 2-fold ($n = 8$, $^{**}p = 0.01$), and 6-fold ($n = 6$, $^{***}p = 0.0007$) increase in TUNEL-positive cells in HONE-1, HONE-1-EBV and HK1-LMP1 cells, respectively (Fig. 2(C)). These results were consistent with previous findings in MKN28 and Colon26 cells [31].

3.3. Cisplatin-resistant NPC cells are sensitive to ECyd

Cisplatin is the most commonly used platinum-based chemotherapy for NPC, with an average response rate of ~55–70% [32]. Since resistance to cisplatin is common in NPC, we examined if this potent antitumor agent can overcome cisplatin-resistance in NPC cell models. As shown in Fig. 3, cisplatin-resistant cell lines derived from the EBV-associated HONE-1-EBV and HK1-LMP1 cell lines (Supplementary Fig. 2), namely HONE-1-EBV-CisR and HK1-LMP1-CisR (clones A–C), were also found to be very sensitive to the growth inhibitory activity of ECyd at nM concentrations (Fig. 3), implicating this RNA-directed anti-metabolite may be useful for treating cisplatin-resistant or cisplatin-non-responsive NPC.

3.4. ECyd induces marked downregulation of TIGAR and NADPH in NPC cells

Thus far, our findings indicate that ECyd exhibits high antitumor potency in NPC cells. A more detailed examination of the effects of ECyd on NPC cell death led us to the unexpected finding that ECyd-induced a marked downregulation of TIGAR, a newly described regulator of apoptosis and glycolysis (*TP53*-induced Glycolysis and Apoptosis Regulator) [33] in NPC cells, which may implicate a novel antitumor mechanism of ECyd. As shown in Fig. 4(A), ECyd-induced a dose-dependent downregulation of TIGAR protein in all 3 NPC cell lines as early as 24 h, which persisted even at 48 h. TIGAR was first identified as a p53 (or *TP53*)-target gene. However, it is believed that p53-independent regulation may also occur in cancer cells (though the detailed mechanism is still unclear at the moment) [33], which is further supported by the recent finding that TIGAR can function in a p53-independent manner [33,34]. Our data suggested that this novel effect of ECyd-induced TIGAR downregulation seemed to be potentially p53-independent as indicated by moderate upregulation of p53 protein at both 24 h and 48 h, but significant downregulation of TIGAR protein levels throughout. Our findings may suggest the involvement of p53-independent regulation of TIGAR triggered by this RNA-directed anti-metabolite.

To further investigate the mechanism of ECyd-induced TIGAR protein downregulation, we examined if transcriptional inhibition of TIGAR was involved. We determined the expression levels of

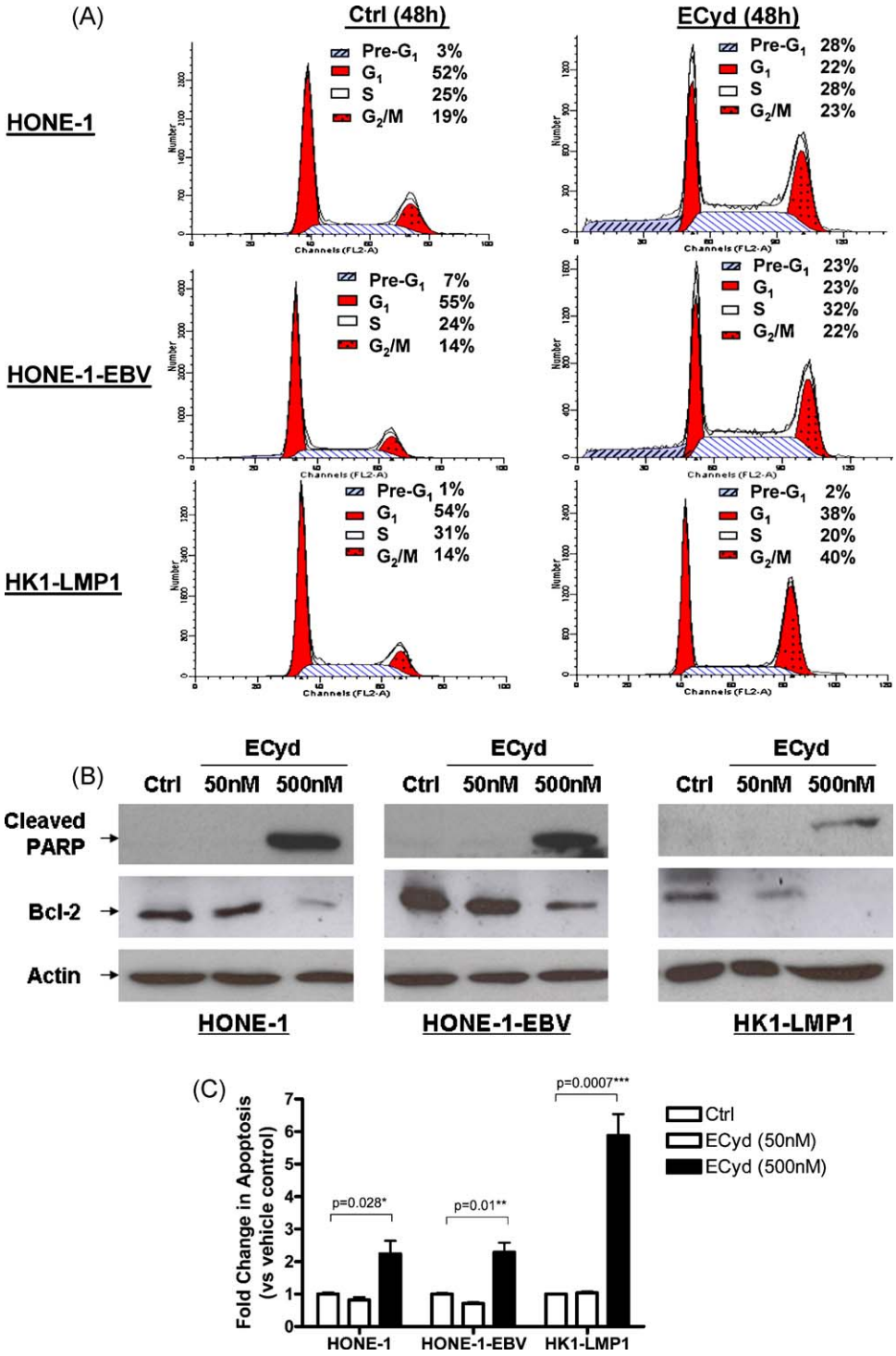

Fig. 2. ECyd induces G_2/M arrest, PARP cleavage and Bcl-2 downregulation in NPC cells. HONE-1, HONE-1-EBV and HK1-LMP1 cells were treated with ECyd (500 nM) or control (medium only) for 48 h. (A) Cell cycle analysis showed G_2/M arrest upon ECyd treatment (48 h) in all 3 cell lines ($n = 3$). Representative original cell cycle histograms were shown for each cell line. (B) Representative Western blot results showing PARP cleavage and Bcl-2 downregulation in all 3 NPC cell lines upon ECyd treatment. Beta-actin was used as a control. Consistent results were observed in three independent experiments. (C) TUNEL ELISA assay demonstrated a significant increase in TUNEL-positive cells upon ECyd treatment (at 48 h) in all 3 NPC cell lines. Cumulative data from 3 independent experiments were shown for each cell line. Apoptosis induced by ECyd was presented as fold change in apoptosis (vs vehicle control) = OD (405–490 nm)$_{ECyd}$/OD (405–490 nm)$_{vehicle}$.

TIGAR mRNA, as well as that of p53 mRNA in these NPC cells upon ECyd treatment. ECyd treatment (at 500 nM) resulted in a marked reduction of TIGAR mRNA expression in both HONE-1 and HONE-1-EBV at 48 h post-treatment, when compared to the respective vehicle control. In HK1-LMP1 cells, a marked reduction of TIGAR mRNA expression was observed more prominently at an earlier time point (24 h) (Fig. 4(B)). As marked TIGAR protein down-regulation was consistently observed at both 24 h and 48 h in all 3 NPC cells (Fig. 4(A)), our collective data indicated that ECyd seemed to downregulate TIGAR at both translational (at protein level as early as 24 h) and transcriptional levels (ranging from 24 h to 48 h). Interestingly, the level of p53 mRNA expression was not significantly altered in any of these NPC cell lines tested, which

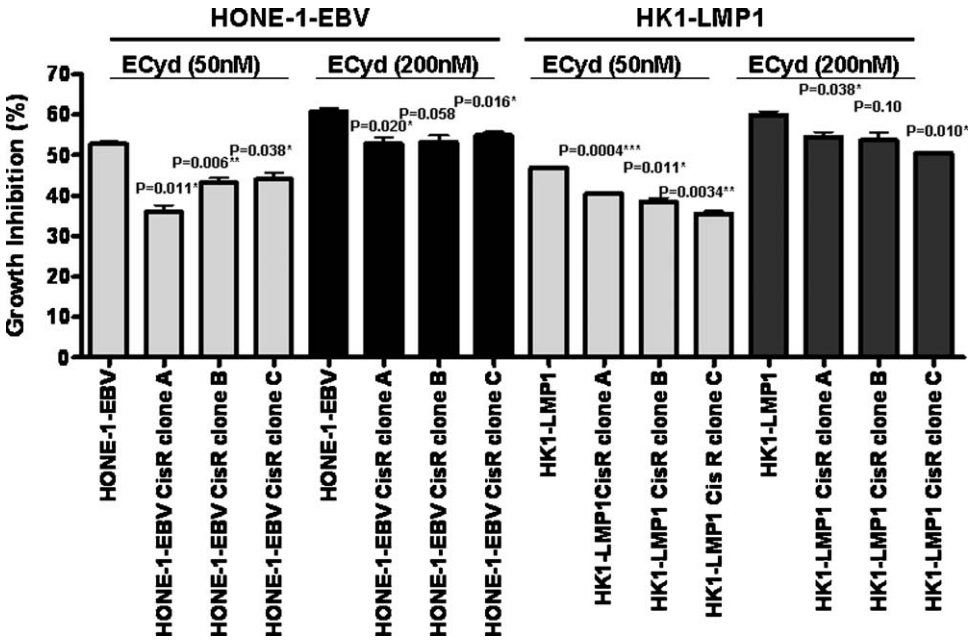

Fig. 3. Cisplatin-resistant NPC cell lines were sensitive to ECyd treatment at nM concentration. Cisplatin-resistant NPC cell lines (HONE-1-EBV CisR and HK1-LMP1 CisR, clones A–C) were derived from the respective parental HONE-1-EBV and HK1-LMP1 cell lines by long-term selection under cisplatin. All cell lines were treated with ECyd (50 nM and 200 nM, indicated by grey and closed bars, respectively) for 48 h. MTT assay was performed to evaluate the effects of ECyd on cell growth ($n = 3$, p-values were shown for each cisplatin-resistant cell line in comparison with the respective parental NPC cell lines).

further demonstrated the specificity of ECyd-induced TIGAR downregulation in NPC cells.

TIGAR has been postulated to inhibit apoptosis via regulation of cellular NAPDH levels (through modulation of the pentose phosphate pathway) [33]. Our finding that ECyd significantly downregulated TIGAR expression in NPC cells suggested its potential effect on cellular NADPH production. As shown in Fig. 4(C), ECyd treatment did induce a significant reduction (up to ~80%, $p < 0.001$) of intracellular NADPH in all 3 NPC cells lines at 48 h, when compared to vehicle. Similar effects were seen as early as 24 h (data not shown). Our result provides the first demonstration in human cancer cells that this new class of RNA-directed antimetabolite could result in significant reduction of cellular NADPH, the key reducing power in mammalian cells. This novel finding was consistent with the recent study by Bensaad et al. that specific downregulation of TIGAR by siRNA induced cancer cell death by altering the pentose phosphate pathway [33], which is the major pathway regulating the production of cellular NADPH.

3.5. TIGAR overexpression reverses ECyd-induced growth inhibition

Since ECyd-induced TIGAR downregulation was accompanied by reduction of NADPH levels in NPC cells, we hypothesized that TIGAR downregulation may represent a novel mechanism for ECyd-induced growth inhibition of NPC cells. Thus, we performed a rescue experiment to examine if overexpression of TIGAR could reverse the growth inhibitory activity of ECyd in NPC cells. Using two transfectable NPC cell line, HONE-1-EBV and the parental HONE-1 (HK1-LMP1 was not transfectable), we found that transient overexpression of TIGAR was able to rescue NPC cells from ECyd-induced growth inhibition (Fig. 5). In vector transfected HONE-1 cells, ECyd (500 nM) inhibited cell proliferation by $37 \pm 1\%$ at 24 h, while transient expression of TIGAR reduced the growth inhibitory effect of ECyd to only $15 \pm 0\%$ ($^{**}p = 0.0075$). Similar result was observed in HONE-1-EBV cells, except that the maximal effect was observed at 48 h. Overexpression of TIGAR significantly reversed the growth inhibitory activity of ECyd in HONE-1-EBV cells (from $44 \pm 2\%$ down to $5 \pm 0\%$, $^{**}p = 0.0030$), indicating TIGAR is involved in ECyd-mediated antitumor activity. Our results demonstrated the direct involvement of TIGAR in ECyd-mediated growth inhibition in NPC cells.

4. Discussion

ECyd is a promising anticancer ribose sugar-modified nucleoside analog, which has recently entered Phase II clinical trial in head and neck cancers for efficacy evaluation. Our current study demonstrated that ECyd effectively (with low IC_{50} values of 5–43 nM) inhibited cancer cell proliferation of NPC origin, which is a highly invasive and metastatic Asian-prevalent head and neck cancer. We also demonstrated that cisplatin-resistant NPC cells were sensitive to ECyd (at nM concentration). The ECyd-mediated growth inhibition was associated with G_2/M cell cycle arrest, PARP cleavage (hallmark of apoptosis) and Bcl-2 downregulation, implicating effective induction of apoptosis by ECyd in NPC cells. Unexpectedly, ECyd was found to downregulate TIGAR, a newly described dual regulator of apoptosis and glycolysis. This novel action of ECyd on TIGAR was associated with marked reduction of NADPH, a major reductive power critically required for cellular proliferation and survival. This suggests a potential mechanistic involvement of TIGAR down-modulation in the antitumor action of ECyd. Indeed, overexpression of TIGAR reversed the growth inhibitory effects of ECyd in NPC cells, demonstrating a new antitumor mechanism of this promising clinical trial agent in human cancer.

Previous studies showed that ECyd inhibits tumor cell proliferation via activation of RNase L-mediated RNA degradation pathway, activation of caspase-dependent DNA fragmentation with subsequent induction of apoptosis, as well as downregulation of HIF-1α in hypoxic tumor cells [21]. Data from our NPC study demonstrated a novel antitumor mechanism of ECyd via TIGAR downregulation. TIGAR has been postulated to inhibit apoptosis via regulation of cellular NAPDH levels (through modulation of the pentose phosphate pathway) [33]. Bensaad et al. have recently demonstrated that specific downregulation of TIGAR (by siRNA) was able to induce cancer cell death by altering the pentose

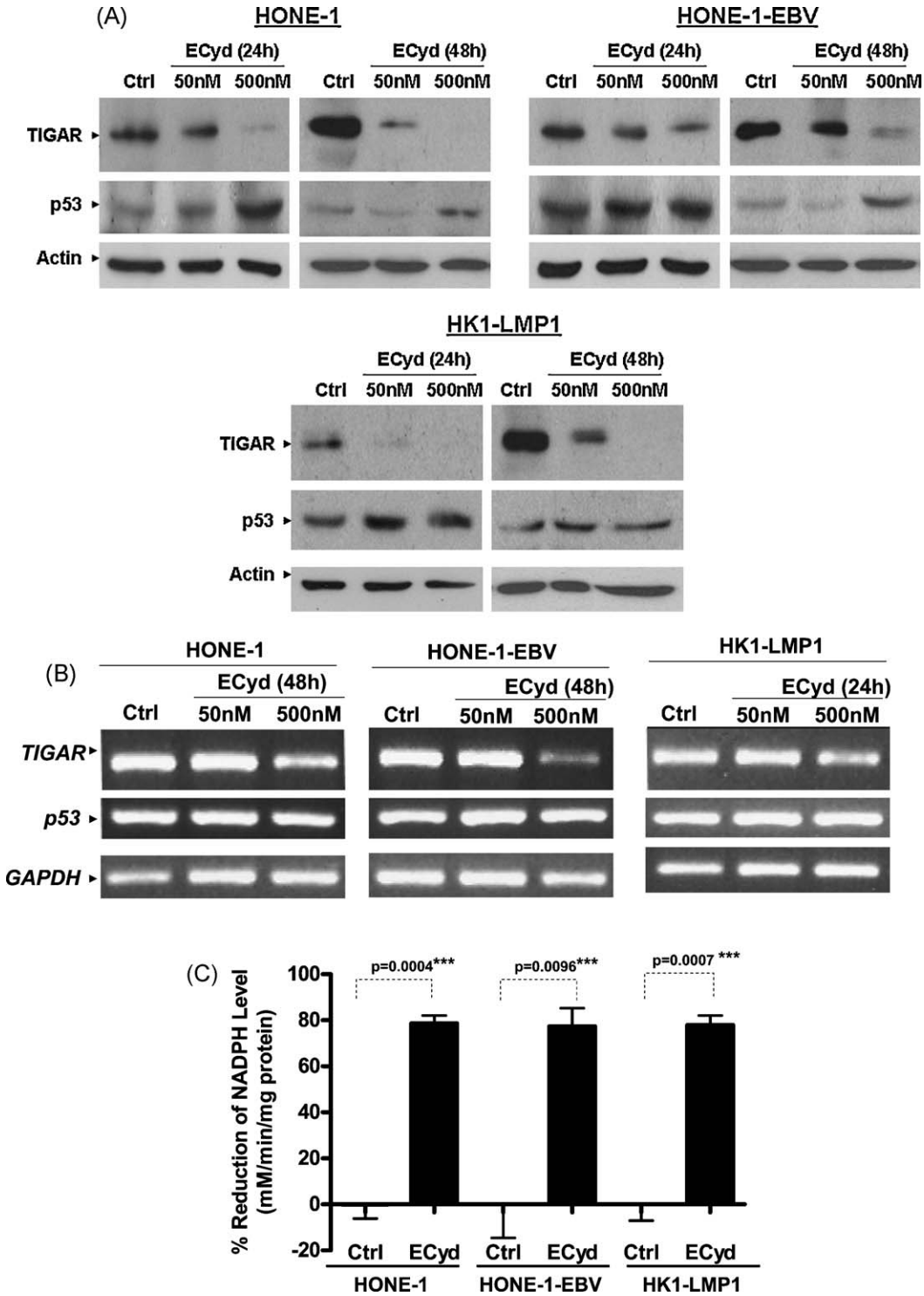

Fig. 4. A. ECyd induces marked downregulation of TIGAR in all 3 NPC cell lines. HONE-1, HONE-1-EBV and HK1-LMP1 cells were treated with ECyd (50 nM and 500 nM) or control (medium only) for 24 h and 48 h. ECyd treatment caused a significant downregulation of TIGAR at nM concentration, while p53 was moderately induced. Beta-actin was used as a control. Similar results were obtained in three independent experiments. (B) ECyd induces a specific inhibition of TIGAR mRNA expression (by RT-PCR), but not p53 mRNA expression in NPC cells. The expression levels of both TIGAR and p53 mRNA in NPC cells were examined using specific primers for RT-PCR (as detailed in the Materials and Methods section). ECyd treatment (at 500 nM) resulted in a marked reduction of TIGAR mRNA expression in both HONE-1 and HONE-1-EBV at 48 h post-treatment, when compared to the respective vehicle control. In HK1-LMP1 cells, a marked reduction of TIGAR mRNA expression was observed more prominently at early time point (24 h). p53 mRNA levels were not altered in any of these cell lines. GAPDH was shown as loading control. Similar results were obtained in $\geq$3 independent experiments. (C) ECyd depletes NAPDH in NPC cells. Cumulative results for percentage change in NADPH levels at 48 h were shown ($p < 0.001$, $n = 4$).

phosphate pathway [33], which is the major pathway regulating the production of cellular NADPH. Our observed ECyd-mediated TIGAR downregulation in NPC cells was found to be associated with NADPH reduction, which is consistent with these previous findings. This is the first demonstration that an RNA-directed anti-metabolite, ECyd, was able to alter cancer cell metabolism at the level of NADPH. It is likely that ECyd-induced depletion of cellular NADPH may severely limit the heavy demands of reductive biosynthesis of DNA, RNA, fatty acid and cholesterol in these rapidly proliferating cancer cells, thus resulting in cell death.

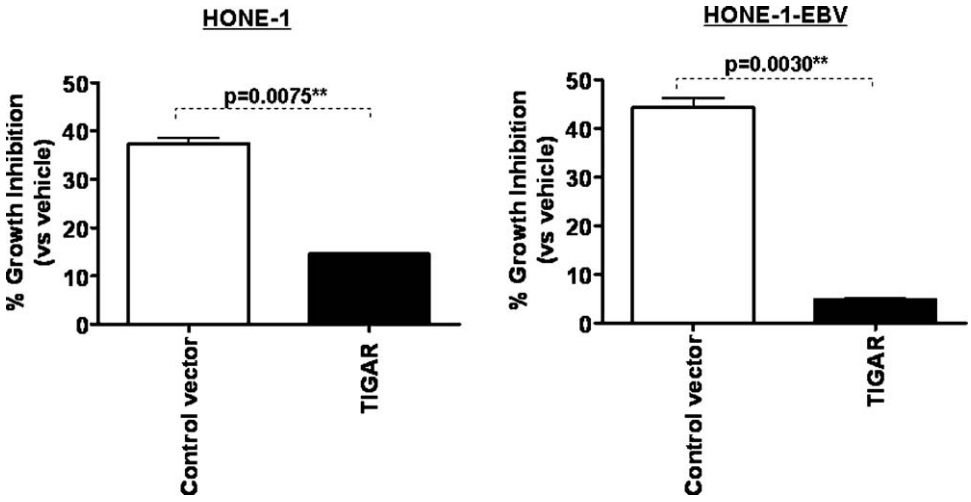

Fig. 5. Overexpression of TIGAR reverses the growth inhibitory activity of ECyd in NPC cells. Both HONE-1 and HONE-1-EBV (HK1-LMP1 was not transfectable) were plated overnight (at 1.4×10^4 cells/well in a 24-well plate) and then transfected with 0.5 μg of control vector (open bar) or TIGAR expression plasmid (closed bar) for an additional 24 h. Transfected cells were treated with control (medium only) or ECyd (500 nM) for indicated periods of time, followed by MTT assay. For HONE-1 and HONE-1-EBV, maximal effects of TIGAR transfection on ECyd activity were observed at 24 h and 48 h, respectively.

Therefore, in addition to directly inhibiting RNA synthesis or active transcription in cancer cells, ECyd may exert a more global constraint on cancer cells by limiting DNA, RNA, fatty acid and cholesterol synthesis via NADPH depletion. This may partly explain the previous unaccounted inhibitory effect of ECyd on DNA synthesis, as the long-postulated inhibitory effect of ECyd on ribonucleoside diphosphate reductase (an enzyme required for the synthesis of DNA precursor) remains unproven [7,14,35].

The exact detailed mechanism for ECyd-induced TIGAR downregulation is currently under investigation as it may provide an important link between RNA metabolism and TIGAR in cancer. TIGAR, as its name suggests, was first described as a downstream inducible gene of the major tumor suppressor gene, *TP53* (or p53 protein). Interestingly, a recent study by Bensaad et al. [33] showed that TIGAR may also be potentially regulated by p53-independent mechanism. Evidence from this current study seems to suggest that ECyd-mediated TIGAR downregulation may occur via p53-independent mechanism as the observed TIGAR downregulation was not accompanied by p53 downregulation, but unexpectedly p53 induction upon ECyd treatment (Fig. 4(A)). The induction of p53 seems to agree with the reported effects of most transcriptional inhibitors [4]. However, the detailed mechanism for this potential p53-independent TIGAR downregulation was not clear at the moment and requires further in-depth investigation. Especially, Bensaad et al. showed that mutated p53 was still capable of inducing TIGAR expression in a human epithelial-like osteosarcoma cell line, SAOS2 cells [33], it is rather unclear how p53 mutation may alter TIGAR regulation in cancer as some NPC harbors p53 mutation, although not very frequently [36]. Although the effect of p53 mutation on TIGAR regulation is yet-to-be investigated in detail, nevertheless, data from our study suggest that the ability of ECyd to down-modulate TIGAR, thus cell death, was still intact despite possible p53 mutation in NPC cells.

NADPH, produced mainly by the pentose phosphate pathway, is not only the key reducing power for reductive biosynthesis of important biomolecules for cell survival and proliferation, but also critically required for cellular protection from oxidative or ROS-mediated damage [37,38]. In the presence of NADPH, oxidized glutathione (GSSG) is converted to reduced glutathione (GSH, a tripeptide with a free sulfhydryl group), which effectively combats against oxidative stress and maintains the normal reduced state in the cell [39]. Thus, the observed ECyd-induced NADPH depletion may also cause potential depletion of GSH in cancer cells, thus making them more prone to ROS-mediated or oxidative damage. This may explain the previously observed radiosensitization effect of ECyd treatment in gastric adenocarcinoma [31]. Whether ECyd can induce radiosensitivity in NPC will warrant further investigation as radiotherapy is commonly used in NPC treatment.

Cisplatin is a DNA-crosslinking agent widely used for the treatment of recurrent or metastatic NPC. However, resistance to cisplatin is frequently developed in NPC patients. Our finding that cisplatin-resistant NPC cells remain highly sensitive to this new ribonucleoside analog is encouraging as these two drugs have very different mechanisms of action. Further preclinical and clinical investigations may help establishing ECyd as a new therapy option for cisplatin-resistant or cisplatin-non-responsive NPC patients. In fact, the on-going Phase II clinical trial of ECyd in recurrent or metastatic head and neck cancer refractory to platinum-based chemotherapy may provide important insight for such an indication.

As a conclusion, our study demonstrated the therapeutic potential of this promising RNA-directed anti-metabolite in NPC. We also revealed for the first time a novel antitumor mechanism of ECyd in inhibiting human cancer cell growth via TIGAR down-modulation. The study may implicate a novel link between RNA metabolism and TIGAR regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.02.012.

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