Review Article

Biochemical Modulation of Aracytidine (Ara-C) Effects by GTI-2040, a Ribonucleotide Reductase Inhibitor, in K562 Human Leukemia Cells

Ping Chen,¹ Josephine Aimuwu,¹ Zhiliang Xie,¹ Xiaohui Wei,¹ Shujun Liu,^{2,3} Rebecca Klisovic,^{2,3,4} Guido Marcucci,^{1,2,3,4} and Kenneth K. Chan^{1,3,4,5}

Received 3 August 2010; accepted 3 December 2010; published online 30 December 2010

Abstract. GTI-2040 is a potent antisense to the M2 subunit of the ribonucleotide reductase (RNR), an enzyme involved in the *de novo* synthesis of nucleoside triphosphates. We hypothesized that combination of GTI-2040 with the cytarabine (Ara-C) could result in an enhanced cytotoxic effect with perturbed intracellular deoxynucleotide/nucleotide (dNTP/NTP) pools including Ara-C triphosphate (Ara-CTP). This study aims to provide a direct experimental support of this hypothesis by monitoring the biochemical modulation effects, intracellular levels of Ara-CTP, dNTPs/NTPs following the combination treatment of Ara-C, and GTI-2040 in K562 human leukemia cells. GTI-2040 was introduced into cells via electroporation. A hybridization–ligation ELISA was used to quantify intracellular GTI-2040 concentrations. Real-time PCR and Western blot methods were used to measure the RNR M2 mRNA and protein levels, respectively. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay was used to measure the cytotoxicity following various drug treatments. A non-radioactive HPLC-UV method was used for measuring the intracellular Ara-CTP, while a LC-MS/MS method was used to quantify intracellular dNTP/NTP pools. GTI-2040 was found to downregulate M2 mRNA and protein levels in a dose-dependent manner and showed significant decrease in dNTP but not NTP pool. When combining GTI-2040 with Ara-C, a synergistic cytotoxicity was observed with no further change in dNTP/NTP pools. Importantly, pretreatment of K562 cells with GTI-2040 was found to increase Ara-CTP level for the first time, and this effect may be due to inhibition of RNR by GTI-2040. This finding provides a laboratory justification for the current phase I/II evaluation of GTI-2040 in combination with Ara-C in patients with acute myeloid leukemia.

KEY WORDS: Ara-CTP; GTI-2040; HPLC-UV.

INTRODUCTION

Ribonucleotide reductase (RNR), a highly regulated enzyme involved in the *de novo* synthesis of 2'-deoxyribonucleotides, plays a critical role in nucleoside metabolism (1,2). RNR catalyzes the reduction of ribonucleotides (ADP, GDP, UDP, and CDP) to their corresponding deoxyribonucleotides (dADP, dGDP, dUDP, and dCDP), and this process is the rate-limiting step required for DNA replication (3). Human RNR consists of two subunits. The M1 subunit contains a substrate binding site, an allosteric site, and a redox active disulfide. The

M2 subunit contains an oxygen-linked non-heme iron center and a tyrosine residue. Both M1 and M2 subunits are essential for catalytic activity (4,5). M2 protein is only expressed during the late G1/early S phase essential for DNA synthesis and repair, while M1 protein level remains relatively stable throughout the cell cycle (5). It has been found that overexpression of M2 protein is associated with malignant and metastatic status of tumor cells. Inhibition of RNR induces imbalance of ribonucleotide and deoxyribonucleotide levels, leading to the inhibition of DNA synthesis and repair and to the induction of cell cycle arrest and apoptosis (6). For this reason, M2 is an excellent target for anticancer drugs development (7,8).

A number of RNR inhibitors, such as hydroxyurea, gemcitabine, and antisense GTI-2040, have been developed (8,9). GTI-2040, a 20-mer oligonucleotide complementary to the coding region of M2 mRNA with the sequence of 5'-GGCTAAATCGCTCCACCAAG-3', is designed to bind to M2 mRNA, resulting in the recruitment of RNase H which in turn induces the cleavage of the drug–mRNA complex and degradation of the target mRNA. *In vitro* studies have demonstrated that treatment of GTI-2040 in a variety of tumor cell lines, such as human H460 lung carcinoma, human T24 bladder cancer, and murine L cell lines, with GTI-2040 led to a sequence- and target-specific downregulations of M2

Ping Chen and Josephine Aimuwu contributed equally to this work.

¹ College of Pharmacy, The Ohio State University, Columbus, Ohio 43210, USA.

² Division of Hematology–Oncology, The Ohio State University, Columbus, Ohio 43210, USA.

³ College of Medicine, The Ohio State University, Columbus, Ohio 43210, USA.

⁴ The Comprehensive Cancer Center, The Ohio State University, Room 302/308 OSU CCC, 410 W. 12th Avenue, Columbus, Ohio 43210, USA.

⁵ To whom correspondence should be addressed. (e-mail: chan.56@osu.edu)

RNR mRNA and protein levels (7). In mice bearing Burkitt's lymphoma, GTI-2040 treatment greatly increased their survival rate (7). A phase I clinical evaluation of GTI-2040 was conducted (10), and its clinically safe doses were established. Since RNR mediates reduction of ribonucleotides, it is expected that its inhibition by GTI-2040 should result in alteration of intracellular dNTP levels and such could provide potential combination treatment strategies with antimetabolite drugs that modulate DNA synthesis and potentiate their antitumor activity.

Ara-C is a widely used antimetabolite for the treatment of acute leukemia (11,12). Inside the cell, Ara-C needs to be phosphorylated to Ara-C triphosphate (Ara-CTP) by deoxycytidine kinase to compete with dCTP for incorporation into DNA. This incorporation causes DNA synthesis inhibition and cell death (13). If intracellular dNTP levels, especially dCTP, are reduced, an increase in Ara-CTP level is expected, leading into an increased antitumor activity of Ara-C (Fig. 1). Based on this rationale, a phase I study of GTI-2040 in combination with Ara-C for the treatment of acute myeloid leukemia (AML) was carried out at this institution (14). This study has demonstrated that GTI-2040 and Ara-C can be safely given to AML patients (10). However, the experimental verification of their biochemical modulation following combination therapy remains to be illustrated. Herein, we developed a non-radioactive HPLC-UV assay to quantify cellular Ara-CTP levels for evaluation of the combination effect of GTI-2040 with Ara-C in human leukemia cell line K562 in order to provide a direct experimental support for the above rationale. This was coupled to the use of a newly developed sensitive LC-MS/MS method (11) to probe the perturbation of intracellular dNTP pools inside the cells.

Additionally, intracellular levels of GTI-2040, M2 mRNA, and M2 protein were determined to further examine the mechanism of action of GTI-2040. This information will be critical in optimizing dosing regimen for future clinical trials.

METHODS

Chemicals and Reagents

Ara-C 5'-triphosphate was purchased from Jena Bioscience (Jena, Germany). 7-Deaza-2'-deoxyguanosine 5'-triphosphate lithium salt (7-deaza-dGTP), potassium chloride (KCl), and potassium phosphate monobasic (KH_2PO_4) were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Sodium acetate was obtained from Sigma. Deionized water for HPLC analysis was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). GTI-2040 and Ara-C were provided by the National Cancer Institute (Bethesda, MD, USA) and used without further purification.

Cell Culture

The human leukemia cell line K562 was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI 1640 media (Supplied by Tissue Culture Shared Resource, Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA) supplemented with 10% fetal bovine serum (Invitrogen, Rockville, MD, USA), L-glutamine (Invitrogen), and penicillin-streptomycin antibiotics (Gibco, Rockville, MD, USA). The cell line

Fig. 1. Diagrammatic rationale for the combination of GTI-2040 with Ara-C in leukemia cells. GTI-2040 inhibits ribonucleotide reductase (RNR), which causes depletion of dNTP and NTP pools, hence allowing phosphorylated Ara-C (Ara-CTP) to be incorporated into DNA resulting in DNA synthesis inhibition and apoptosis

was maintained at 37°C in a humidified environment with 5% CO₂. Viability and cell counts were determined using trypan blue dye exclusion assay (15). GTI-2040 was initially introduced into cells using a neophectin transfecting agent and later with an electroporation device (Bio-Rad Lab, Hercules, CA, USA).

Determination of Intracellular GTI-2040 Concentrations by a Hybridization-Based ELISA

K562 cells (5×10^6) were treated with GTI-2040 at 0, 1, 5, 10, and 20 μ M for 24 h using an electroporation delivery technique. After 24 h, cells were harvested and intracellular GTI-2040 levels were measured using a previously developed two-step hybridization–ligation ELISA assay (16). Briefly, GTI-2040 was first base-paired to the capture probe in a polypropylene 96-well plate. Ten percent Triton X-100 was added to the mixture solution and incubated at 42°C for 2.5 h for hybridization. The resulting solution was transferred to a NeutrAvidin-coated 96-well plate, which was incubated at 37°C for 30 min to ensure the attachment of biotin-labeled capture probe to NeutrAvidin-coated wells. After washing six times, the ligation solution containing T4 ligase and detection probe was added to each well followed by the addition of S1 nuclease solution. The reaction was blocked with Superblock buffer (Pierce, IL, USA). Anti-digoxigenin–alkaline phosphatase was then added into each well. Following addition of substrate solution (36 mg Attophos in 60 mL diethanolamine buffer), fluorescence intensity was measured at Ex 430/Em 560 (filter=550 nm) using a Gemini XS fluorescence microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

RNA Isolation and RT-qPCR

To measure intracellular M2 mRNA level, 5×10^6 K562 cells were treated with GTI-2040 at 0, 5, 10, and 20 μ M for 24 h. In the combination study, 5×10^6 K562 cells were first treated with GTI-2040 alone at 10 μ M for 24 h followed by continuous treatment with Ara-C at concentrations of 5, 10, and 20 μ M for an additional 48 h, while the GTI-2040 exposure was maintained. Total RNA was isolated using Trizol reagent (Invitrogen). Briefly, cell lysate was treated with chloroform, and the total RNA was precipitated with isopropyl alcohol, followed by a washing step with 75% ethanol. RNA was then dissolved in RNase free water, and its concentration and purity was measured by a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). cDNA was synthesized from 2 μ g total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen). The cDNA templates and primers were then mixed with reagents from a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Reactions were carried out in triplicate in ABI prism 7700 sequence detector (Applied Biosystems), and data were analyzed by comparative CT method. Dissociation curves were also obtained to examine the purity of the amplified products. The amount of M2 mRNA in each sample was normalized with respect to an internal control, *abl*. The relative changes in treated groups were expressed as a

percentage of untreated control (arbitrarily set at 1). The results were expressed as the mean $\pm$ SD from triplicate determinations.

Western Blot Analysis

K562 cells (5×10^6) were treated with GTI-2040 as a single agent at 0, 1, 5, 10, 20, and 30 μ M for 24 h. In the combination study, 5×10^6 K562 cells were first treated with GTI-2040 alone at 10 μ M for 24 h followed by Ara-C continuous exposure at concentrations of 5, 10, and 20 μ M for 48 h. Cells were then harvested, washed with 1 mL ice-cold PBS, and centrifuged at $1,000 \times g$ for 5 min at 4°C. The pellet was obtained and re-suspended in 100 μ L lysis buffer (50 mM Tris–HCl (pH 7.6), 250 mM NaCl, 5 mM EDTA, 2 mM Na₃VO₄, 50 mM NaF, and 1% protease inhibitor cocktail; P8340, Sigma) for 30 min on ice. The lysate was sonicated for 10 s. Total protein concentration was determined using the BCA protein assay method (Pierce, Rockford, IL, USA). Equal amounts of protein for each sample were incubated with 6 $\times$ SDS loading buffer (100 mM Tris (pH 6.8), 200 mM DTT, 4% SDS, 20% glycerol, and 0.015% bromophenol blue) and boiled for 5 min. The proteins were then separated on 15% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (Amersham, Piscataway, NJ, USA). The M2 protein was recognized following treatment with a goat antihuman M2 polyclonal antibody (E-16; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the first antibody, followed by a horseradish peroxidase conjugated anti-goat IgG secondary antibody. M2 protein (MWT 45,000 Da) was detected by ECL (Amersham, Arlington Heights, IL, USA), and GAPDH was used as the internal loading control. M2 protein expressions were quantified by densitometry and normalized to GAPDH.

Determination of Intracellular dNTP and NTP Pools

K562 cells (10×10^6) each were treated either with Ara-C alone at concentrations of 5, 10, and 20 μ M for 24 h, or pretreated with GTI-2040 at 10 μ M and 24 h later treated with continuous exposure of Ara-C at concentrations of 5, 10, and 20 μ M for 48 h. Cells were then lysed and dNTPs/NTPs were extracted and quantified by our previously published method (17). Briefly, cells were counted and monitored for viability using trypan blue exclusion test before extraction. Following centrifugation at $1,000 \times g$ for 5 min, cell pellets were washed with phosphate-buffered saline (PBS) and deprotonized with an addition of 1 mL 60% methanol. The resulting solution was vortex-mixed for 20 s, incubated in -20°C for 30 min, and sonicated for 15 min in an ice bath. Cell extracts were centrifuged at $1,000 \times g$ for 5 min at 4°C, and the supernatant was separated and dried under a stream of nitrogen. The residues were reconstituted with 300 μ L of water, vortex-mixed for 20 s, and the cell extracts were centrifuged at $1,000 \times g$ for 5 min at 4°C. A 50- μ L aliquot of the resulting supernatants was injected into an ion-trap LC-MS/MS system (LCQ, Thermo Scientific, San Jose, CA, USA) for dNTP and NTP measurements.

Growth Inhibition Assay

To evaluate the growth inhibitory effect of GTI-2040 and Ara-C on K562 cells, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cytotoxicity assay was performed. GTI-2040 was introduced into K562 cells by using either neophectin transfecting agent or with an electroporation device. Briefly, cells were seeded into 96-well plates, and the cells were pretreated with fixed concentrations 5 or 10 μ M of GTI-2040 for 24 h. Increasing concentrations of Ara-C at 0.01, 0.1, 1, 10, and 100 μ M were then added, and the incubation was continued for 72 h. In a reverse sequence, K562 cells were pretreated with a fixed concentration 1 μ M of Ara-C for 24 h, then graded concentrations of GTI-2040 at 1, 3, 10, 30, and 100 μ M, were added, and the incubation was continued to 72 h. Afterward, 20 μ L of MTS and phenazine methosulfate (Promega, Madison, WI, USA) was added to each well in a 20:1 ratio. After 2 h incubation, the absorbance was read at 490 nm on a microplate reader Gemini Xs (Molecular Devices, Sunnyvale, CA, USA) to determine levels of formazan product as a measure of cell viability against a non-drug treated control. Determination of IC_{50} values was performed using WinNonLin software (Pharsight, Mountain View, CA, USA). Isobologram analysis was done using the combination index values based on the Chou-Talalay method (18)

HPLC Method for Ara-CTP Determination and Method Validation

Ara-CTP in the cell matrix was separated on a Partisil-10 SAX anion exchange, 4.6 mm $\times$ 25 cm, 10 μ m particle size column (Whatman, Inc., Clifton, NJ, USA) coupled to a 5- μ m SAX hypersil 10 $\times$ 4-mm drop-in guard column (Thermo Scientific, San Jose, CA, USA). An isocratic elution program with a mobile phase consisting of 0.35 M KH_2PO_4 and 0.15 M KCl in ultra-pure water at a flow rate of 1.5 mL/min at room temperature was used for the separation. To assure the stability of cell extracts, the autosampler temperature was set at 4°C throughout the analysis. The absorbance of eluted compounds was monitored at 280 nm by a SPD-10A detector. Peak areas or peak heights were quantitated with EZStart software.

Fig. 2. Intracellular accumulation of GTI-2040 in K562 cells following introduction of GTI-2040 at the indicated concentrations for 24 h by electroporation. Vertical bars represent mean $\pm$ SD from triplicate experiments. * $p < 0.05$; ** $p < 0.01$, compared to concentration at 1 μ M. The rate of increase appeared to be first order with respect to the exposure concentration a regression coefficient of 0.9855

Fig. 3. GTI-2040 reduces M2 mRNA expression in K562 cells alone (a) in a dose-dependent manner. Ara-C had no effect on M2 mRNA expression alone or no further reduction in M2 mRNA expression caused by 10 μ M GTI-2040 (b). M2 mRNA levels were normalized by abl and are presented as the percentage of the untreated control. Vertical bars represent mean $\pm$ SD from triplicate experiments and compared with the control with asterisks showing $p < 0.05$

Fig. 4. GTI-2040 decreases M2 protein expression in K562 cells. a M2 protein levels were decreased by about 60% following GTI-2040 treatment at concentrations >10 μ M. b Ara-C alone had no effect on M2 protein levels, and addition of various concentrations of Ara-C did not contribute to further reduction of M2 protein levels caused by pre-incubation with GTI-2040 at 10 μ M. M2 protein levels were quantified by densitometry and expressed as the ratios over the loading control GAPDH

The linearity was assessed at the concentration range of 0.2 (2 pmol/ 10^6 cell)–50 μ g/mL (500 pmol/ 10^6 cell) of Ara-CTP in cellular matrices. Within-day and between-day accuracy and precision were determined at 0.5 (5 pmol/ 10^6 cell, low-quality control), 5 (50 pmol/ 10^6 cell, medium-quality control), and 50 μ g/mL (500 pmol/ 10^6 cell, high-quality control), and these concentration ranges were chosen to cover the range of Ara-CTP levels in leukemia patients (19–23). The within-day precision was determined with six replicates, and the between-day precision was determined across three QCs at six different days. The accuracy was assessed by comparing the nominal concentrations with the corresponding calculated concentrations based on the calibration curve. The precision of the assay was calculated by comparing the standard deviation (SD) of the determined concentrations. The specificity of assay was evaluated by comparing the retention times of blank cell matrices with that spiked with 10 μ g/mL Ara-CTP and 10 μ g/mL of the internal standard.

Ara-CTP Extraction and Determination

K562 cells were used for this study, based on the previous reports that demonstrated that significant accumulation of Ara-CTP following incubation with Ara-C (24,25);

10×10^6 K562 cells were treated with 10 μ M Ara-C for 0, 1, 2, 3, 4, 6, 8, and 24 h and then at 4 h, treated with 1, 5, 10, and 20 μ M. For combination study, 10×10^6 K562 cells were pretreated with GTI-2040 at 0, 1, 5, 10, and 20 μ M for 24 h followed by the 10 μ M Ara-C treatments for 4 h. After treating with trypan blue dye and counting with a hemocytometer, cells were centrifuged and the harvested pellets were washed three times with cold PBS. Ara-CTP was extracted using the same extraction method for dNTP/NTP as described above. A 50- μ L aliquot of the final resulting supernatant was injected into HPLC-UV system for Ara-CTP measurement.

RESULTS

Determination of GTI-2040 Concentrations in Human Leukemia K562 Cells

In order to confirm the uptake of GTI-2040 into cells, intracellular GTI-2040 levels were determined. Following the exposure of K562 cells to GTI-2040 at 1, 5, 10, and 20 μ M for 24 h, GTI-2040 cellular concentrations were found to be 168.4 ± 66.8 , 373.1 ± 59.5 , $1,074 \pm 66$, and $1,843 \pm 131$ pmol/ 10^6 cells (mean $\pm$ SD, $n=3$), respectively (Fig. 2). Thus, intracellular GTI-2040 content was found to increase with an increase in

Fig. 5. Perturbation of intracellular dNTP/NTP pools by GTI-2040 and Ara-C. K562 cells were treated with GTI-2040 at the indicated concentrations in the absence and presence of GTI-2040. **a** GTI-2040 at 10 μ M reduced levels of dTTP, dATP, and dCTP by 40%, whereas Ara-C showed no effect; **b** GTI-2040 did not change the NTP pools; however, Ara-C at 10 and 20 μ M decreased GTP, CTP, UTP, and dGTP/ATP by 40–60%. dNTP/NTP levels are presented as the percentage of untreated control. Vertical bars are mean $\pm$ SD of triplicate experiments with asterisks showing $p < 0.05$ versus control

extracellular exposure concentrations. The rate of increase appeared to be first order with respect to the exposure concentration a regression coefficient of 0.9855.

GTI-2040 Reduces M2 mRNA Levels in K562 Cells

Following treatment of K562 cells with 10 and 20 μM GTI-2040 for 24 h, M2 mRNA level was significantly decreased by about 50–60% (Fig. 3a) relative to the untreated control. Treatment of these cells with 5, 10, and 20 μM of Ara-C caused no change in M2 mRNA level, and addition of Ara-C (5, 10, and 20 μM) to the 24 h pretreated cells with 10 μM GTI-2040 for an additional 48 h also did not result in further reduction of M2 mRNA (Fig. 3b). Pretreatment with Ara-C followed by GTI-2040 did not cause an enhance reduction in M2 mRNA caused by GTI-2040 (data not shown). These data confirmed that GTI-2040 regulates M2 at the transcriptional level and indicated that M2 RNR is not a target of Ara-C.

GTI-2040 Decreases M2 Protein Expression

To investigate the effects of GTI-2040 on M2 protein expression, K562 cells were exposed to either GTI-2040 alone or in combination with Ara-C. As shown in Fig. 4a, GTI-2040 treatments at 5 μM for 24 h did not alter the expression of M2 protein significantly until the concentrations were increased to 10, 20, and 30 μM and that the expression of M2 protein decreased by 60%. This indicates that the downregulation requires a threshold of GTI-2040 concentrations and was exposure concentration dependent. Treatment of the cells with Ara-C alone at 5, 10, and 20 μM did not cause downregulation of the M2 protein (Fig. 4b).

In the combination study, pretreatment with 10 μM GTI-2040 for 24 h caused a significant reduction in M2 protein as shown above, and subsequent combination treatment with 5, 10, and 20 μM Ara-C did not cause further decrease in M2 protein expression (Fig. 4b), consistent with the expected mechanism of M2 mRNA downregulation. Taken together, these data confirmed that GTI-2040 but not Ara-C regulates M2 protein at both the transcriptional and translational level.

GTI-2040 Perturbs the Intracellular Ribonucleotide (NTPs) and Deoxyribonucleotide (dNTPs) Pools in K562 Cells

Since M2 is required for catalyzing the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleotides (2), we would like to probe the intracellular dNTP/NTP pool changes caused by GTI-2040 (17) or Ara-C alone and by their combination. K562 cells were first exposed to 10 μM GTI-2040 for 24 h, followed by treatment with 10 or 20 μM Ara-C for 48 h. dNTPs and NTPs were extracted and measured by the LC-MS/MS method as described above (17). We have found a significant decrease (~40%) in intracellular dTTP, dATP, and dCTP levels (Fig. 5a) following 10 μM GTI-2040 exposure alone when compared with the untreated control, but with no effect on the NTP pool. No further decrease was apparent with co-treatment with 10 or 20 μM Ara-C; the small variation due to different Ara-C doses was within the statistical errors. Interestingly, GTP, UTP, CTP,

and dGTP/ATP levels were significantly reduced by Ara-C alone (40–60%), but not by GTI-2040 alone or its combination with Ara-C (Fig. 5b). The lack of change for the latter may be due to the M2 RNR inhibition of GTI-2040.

MTS Assay

Effect of GTI-2040 on Ara-C cytotoxicity in K562 cells was examined following 5 or 10 μM GTI-2040 pretreatment. As shown in Fig. 6a, cytotoxicity of Ara-C alone was observed with an estimated IC_{50} of 0.13 μM . Following preincubation with GTI-2040 at 5 and 10 μM for 24 h, IC_{50} values of Ara-C were reduced to 0.014 μM (a 9.3-fold increase in cytotoxicity) and 0.012 μM (a 10.8-fold increase in cytotoxicity), respectively. These data were assessed for synergism by the method of Chou and Talalay (18), and the resulting combination indices were all found to be <1 , indicating that their combination generates synergistic effects. Cytotoxicity of their combination in the reverse sequence was also evaluated in K562 cells. As shown in Fig. 6b, co-treatment of K562 cells with Ara-C and GTI-2040 with preincubation of Ara-C at 1 μM reduced the IC_{50} values to 0.00076 μM (a 171-fold increase in cytotoxicity compared to Ara-C alone). These data suggest that there were synergistic

Fig. 6. Effect of GTI-2040 on cytotoxicity of Ara-C in K562 cells. **a** Pretreatment of 5 or 10 μM GTI-2040 via electroporation decreased the IC_{50} of Ara-C and showed a combination index of <1 , indicating synergism; **b** pretreatment of 1 μM Ara-C followed by the GTI-2040 treatment via neoplectin also decreased the IC_{50} of Ara-C with a combination index of 0.1, indicating profound synergism (all comparisons showed $*p < 0.05$ versus control)

effect between GTI-2040 and Ara-C which may be more effective when Ara-C treatment precedes GTI-2040.

HPLC Determination of Ara-CTP and Its Method Validation

As shown in Fig. 7, Ara-CTP and its internal standard 7-deaza-dGTP were baseline-resolved from all other intracellular dNTP and NTPs in the cell lysate. The retention times for UTP, dTTP, CTP, dCTP, Ara-CTP, ATP, dATP, GTP, and dGTP and 7-deaza-dGTP (I.S.) were 12.3, 13.7, 13.8, 14.4, 16.3, 20.8, 26.7, 32.9, 37.8, and 42.2 min, respectively. Ara-CTP and 7-deaza-dGTP were identified by their retention times and peak enhancement with spiking of the respective analytes into the cell extracts.

Excellent linearity of the Ara-CTP assay was found between 0.5 $\mu\text{g/mL}$, the lower limit of quantification (LLOQ), and 50 $\mu\text{g/mL}$ in K562 cell lysate, using a 50- μL sample injection. The within-day coefficients of variation (CVs) for Ara-CTP were 19%, 3.5%, and 4.2% at 0.50, 5, and 50 $\mu\text{g/mL}$, respectively. The between-day CVs were 6.8% at LLOQ and 0.6–1.6% between 5 and 50 $\mu\text{g/mL}$ for Ara-CTP. The within-day

accuracy values for Ara-CTP were 105.5%, 100.1%, and 97.2% at 0.50, 5, and 50 $\mu\text{g/mL}$, respectively, based on six replicates.

Time- and Dose-Dependent Accumulation of Ara-C in K562 Cells

In order to monitor the accumulation of Ara-C, the concentration-time course of Ara-CTP in K562 cells following its treatment was followed (Fig. 8a). As shown, Ara-CTP level peaked at 4 h and sustained to 24 h monitored. The accumulation level of Ara-CTP occurred in a dose-dependent manner following Ara-C treatment (Fig. 8b).

Quantification of Intracellular Ara-CTP Levels Following Ara-C Treatment in Combination with GTI-2040 in K562 Cells

Following Ara-C treatment in combination with various concentrations of GTI-2040 in K562 cells, intracellular Ara-CTP levels were found to increase significantly (Fig. 8c). Pretreatment with GTI-2040 at 10 and 20 μM caused an

Fig. 7. **a** A representative HPLC chromatogram of K562 cell lysate extract; **b** a representative HPLC chromatogram of K562 cell lysate extract spiked with 10 $\mu\text{g/mL}$ each of Ara-CTP and 7-deaza-dGTP

Fig. 8. Ara-CTP accumulation in K562 cells. **a** Time-dependent accumulation of Ara-CTP (peaked at ~4 h); **b** dose-dependent accumulation of Ara-CTP following 4 h treatment. Double asterisk shows $p < 0.01$ versus 1 μM as the control; **c** Ara-CTP accumulations w/o GTI-2040 pre-incubation. Cells were pretreated with GTI-2040 at indicated concentrations for 24 h. Cells were then washed and incubated with 10 μM Ara-C for 4 h. Percentage of Ara-CTP accumulation are shown as means $\pm$ standard deviation of triplicate experiments with asterisk showing $p < 0.05$ versus Ara-C alone

increase in Ara-CTP levels by about 50% compared to Ara-C treatment alone, although no significant change in Ara-CTP levels was seen below 10 μM GTI-2040 exposures.

DISCUSSION

In this study, the biochemical modulation effects of Ara-C by GTI-2040 in K562 human leukemia cells were investigated. There are several human leukemia cell lines avail-

able, and only K562 cell line was selected to mimic the clinical situation, since this cell line was found to have higher baseline levels of M2 expression (data not shown), which is similar to those responders of AML patients treated with GTI-2040 and high-dose Ara-C (14). Ara-C is one of the most effective anticancer agents for the treatment of AML, and its therapeutic effect at low or high doses has been extensively studied (24–31). It has been reported that the metabolism of Ara-C *in vitro* was greatly enhanced by RNR inhibitors, such as amidox and trimidox (32–34). Clinical combination studies of Ara-C with RNR inhibitors such as fludarabine and chlorodeoxyadenosine demonstrated significant accumulation of Ara-CTP in lymphocytes and sustained inhibition of DNA synthesis in circulating leukemia blasts from patients with leukemia (35–37). Thus, it appears that inhibition of RNR could result in inhibition of *de novo* dNTP synthesis, enhance Ara-C sensitivity, and even overcome Ara-C resistance (38,39). However, these RNR inhibitors are primarily small molecules and possess other pharmacological effects. As an antisense RNR inhibitor, GTI-2040 may provide more advantages in combination treatment with Ara-C. First, GTI-2040 is less toxic with its $\text{IC}_{50} > 100 \mu\text{M}$ in human leukemia K562 cells. Phase I studies of GTI-2040 alone for the treatment of advanced solid tumors or lymphoma indicated that GTI-2040 was generally well tolerated as a single agent with only two patients experiencing a dose-limiting reversible hepatic toxicity (10). Therefore, GTI-2040 will unlikely result in significant adverse effect in combination with Ara-C. Second, GTI-2040 is stable and efficiently inhibits RNR expression through direct base pairing to M2 mRNA, leading to RNR downregulation. On the contrary, conventional deoxyadenosine analogs such as fludarabine and clofarabine are susceptible to enzymatic phosphorolysis by *Escherichia coli* purine nucleoside phosphorylase and are acid labile. Taken together, GTI-2040 holds promise in combination treatment with Ara-C for human leukemia.

Initially, GTI-2040 was introduced into K562 cells by a neophectin transfecting agent, but later it was found that this method presents several major problems. First, it was found that neophectin and oligofectamine both possess substantial cytotoxicity (with EC_{50} values of 4.2 ± 0.6 and $3.3 \pm 0.6 \mu\text{M}$, respectively). This activity will interfere with the cytotoxicity assessment of GTI-2040 alone and in combination with Ara-C and confound the data interpretation. Second, treatment with transfecting agents also caused a significant interference with the LC-MS/MS measurement of dNTP/NTP pools. We have subsequently found a significant uptake of GTI-2040 via electroporation at about 60–70%, which is comparable to that using a transfecting agent (15) with negligible cytotoxicity itself as a control. For this reason, we employed electroporation for GTI-2040 introduction in all subsequent studies. This is consistent with the finding of Iversen *et al.* (40) which reported electroporation being the preferred method over lipofection method in delivery of gene or gene products, although none of the above methods has been approved for clinical application.

Following GTI-2040 delivery, our results showed significant dose-dependent downregulation of M2 mRNA and protein in K562 cells. These effects provide an additional experimental validation of the proposed mechanism of GTI-2040 in leukemia cells (7). Our data also confirmed that when

the intracellular M2 expression was reduced by GTI-2040, downstream dNTP pool was perturbed, which may enhance Ara-C's incorporation into DNA (41,42).

Our data showed a significant decrease in intracellular dTTP, dATP, and dCTP levels following treatment with GTI-2040 alone or in combination with Ara-C as expected with no change in NTP levels. Interestingly, the GTP, UTP, CTP, and dGTP/ATP pools were significantly reduced by treatment with Ara-C alone (40–60%), and this may be due to salvage or feedback regulations (Ara-CTP conceived as CTP). Alternatively, this could be due to its modulation of synthetases (e.g., CTP synthetase), as it was shown that some pyrimidine antimetabolites could inhibit CTP synthetase causing decrease of CTP level (43). This enzyme catalyzes the formation of CTP from UTP and plays a role in the determination of cellular CTP and dCTP pools (43,44). Possibly, Ara-C inhibits CTP synthetase and, concomitantly, disrupts both the intracellular CTP and dCTP.

Our study also demonstrated a synergistic effect in cytotoxicity in K562 cells with combination of GTI-2040 and Ara-C. The extent of the synergistic effects was more significant when Ara-C treatment preceded the treatment with GTI-2040. Possibly, this further enhancement could be due to the first incorporation of Ara-CTP into DNA to compete with dCTP causing immediate chain termination and blockade of DNA synthesis (45). Further addition of GTI-2040 may result in an enhanced depletion of dCTP pool, a sustained higher cellular Ara-CTP levels, and thus potentiation of cytotoxicity. Despite these differences, in both treatment orders, we have demonstrated that GTI-2040 enhanced the cytotoxicity of Ara-C in leukemia cells. Based on the cytotoxicity results, alternative sequencing schedules for the combination should be considered in future clinical trial design with careful attention for relevant pharmacodynamic endpoints.

A non-radioactive HPLC-UV method was optimized in our study to quantify the Ara-CTP level in the cell. We first evaluated an ion-pair HPLC method developed by Schilsky and Ordway (46). However, severe baseline drift during sample analysis hampered the use of this method. Then, we adapted the method developed by Plunkett *et al.* (47), using our Shimadzu HPLC system with non-radioactive Ara-CTP. However, we were unable to resolve CTP, dCTP, and Ara-CTP satisfactorily probably due to differences in the use of a different HPLC system (Waters Associate for the published method). After several attempts, we successfully separated Ara-CTP from dNTPs and NTPs using an isocratic elution program with a mobile phase consisting of 0.35 M KH_2PO_4 and 0.15 M KCl. Notably, none of the published quantification methods for intracellular Ara-CTP utilized an internal standard, which is essential to compensate sample loss during sample workup. Hence, we sought to use a suitable internal standard and found 7-deaza-dGTP to be satisfactory. As shown in Fig. 6b, 7-deaza-dGTP was baseline-resolved from Ara-CTP and all other intracellular nucleotides and interference substances (Fig. 7). This method was then applied to analyze intracellular Ara-CTP level following Ara-C treatment, which showed time- and dose-dependent Ara-CTP accumulation (Fig. 8a, b, respectively). Pretreatment with GTI-2040 at 10 or 20 μM , followed by Ara-C exposure, led to significant enhancement of intracellular Ara-CTP level supporting the role of GTI-2040 potentiation of Ara-C (Fig. 8c).

CONCLUSION

GTI-2040 was found to inhibit ribonucleotide reductase by downregulation of M2 mRNA and protein levels. The inhibition of ribonucleotide reductase by GTI-2040 results in an increase in Ara-CTP levels, which was found for the first time. Combination of GTI-2040 with Ara-C produced an increase in cytotoxicity. This provides a laboratory and mechanistic justification for the current phase I/II evaluation of GTI-2040 in combination with Ara-C in patients with acute myeloid leukemia.

Acknowledgment This work was partially supported by National Cancer Institute/National Institute of Health grant R21 CA133879 (GM/RBK) and grant UO1-CA76576 and Lorus Therapeutics.

Conflict of interest statement Lorus Therapeutics provided partial support for the development of the Ara-CTP and dNTP/NTP assays.

REFERENCES

1. Fontecave M. Ribonucleotide reductases and radical reactions. *Cell Mol Life Sci.* 1998;54(7):684–95.
2. Reichard P, Ehrenberg A. Ribonucleotide reductase—a radical enzyme. *Science (New York, NY).* 1983;221(4610):514–9.
3. Jordan A, Reichard P. Ribonucleotide reductases. *Annu Rev Biochem.* 1998;67:71–98.
4. Kolberg M, Strand KR, Graff P, Andersson KK. Structure, function, and mechanism of ribonucleotide reductases. *Biochim Biophys Acta.* 2004;1699(1–2):1–34.
5. Nocentini G. Ribonucleotide reductase inhibitors: new strategies for cancer chemotherapy. *Crit Rev Oncol Hematol.* 1996;22(2):89–126.
6. Cory JG. Ribonucleotide reductase as a chemotherapeutic target. *Adv Enzyme Regul.* 1988;27:437–55.
7. Lee Y, Vassilakos A, Feng N, Lam V, Xie H, Wang M, *et al.* GTI-2040, an antisense agent targeting the small subunit component (R2) of human ribonucleotide reductase, shows potent antitumor activity against a variety of tumors. *Cancer Res.* 2003;63(11):2802–11.
8. Smith BD, Karp JE. Ribonucleotide reductase: an old target with new potential. *Leuk Res.* 2003;27(12):1075–6.
9. Bianchi V, Borella S, Calderazzo F, Ferraro P, Chieco Bianchi L, Reichard P. Inhibition of ribonucleotide reductase by 2'-substituted deoxycytidine analogs: possible application in AIDS treatment. *Proc Natl Acad Sci USA.* 1994;91(18):8403–7.
10. Desai AA, Schilsky RL, Young A, Janisch L, Stadler WM, Vogelzang NJ, *et al.* A phase I study of antisense oligonucleotide GTI-2040 given by continuous intravenous infusion in patients with advanced solid tumors. *Ann Oncol.* 2005;16(6):958–65.
11. Estey EH. Therapeutic options for acute myelogenous leukemia. *Cancer.* 2001;92(5):1059–73.
12. Ravindranath Y. Recent advances in pediatric acute lymphoblastic and myeloid leukemia. *Curr Opin Oncol.* 2003;15(1):23–35.
13. Creasey WA, Papac RJ, Markiw ME, Calabresi P, Welch AD. Biochemical and pharmacological studies with 1-beta-D-arabinofuranosylcytosine in man. *Biochem Pharmacol.* 1966;15(10):1417–28.
14. Klisovic RB, Blum W, Wei X, Liu S, Liu Z, Xie Z, *et al.* Phase I study of GTI-2040, an antisense to ribonucleotide reductase, in

- combination with high-dose cytarabine in patients with acute myeloid leukemia. *Clin Cancer Res.* 2008;14(12):3889–95.
15. Wei X. Pharmacokinetics, pharmacodynamics and metabolism of GTI-2040, a phosphorothioate oligonucleotide targeting R2 subunit of ribonucleotide reductase. Columbus: Ohio State University; 2006. <http://rave.ohiolink.edu/etdc/view?acc%5Fnum=osu1143220921>.
16. Wei X, Dai G, Marcucci G, Liu Z, Hoyt D, Blum W, *et al.* A specific picomolar hybridization-based ELISA assay for the determination of phosphorothioate oligonucleotides in plasma and cellular matrices. *Pharm Res.* 2006;23(6):1251–64.
17. Chen P, Liu Z, Liu S, Xie Z, Aimiwu J, Pang J, *et al.* A LC-MS/MS method for the analysis of intracellular nucleoside triphosphate levels. *Pharm Res.* 2009;26(6):1504–15.
18. Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul.* 1984;22:27–55.
19. Avramis VI, Biener R, Krailo M, Finklestein J, Ettinger L, Willoughby M, *et al.* Biochemical pharmacology of high dose 1-beta-D-arabinofuranosylcytosine in childhood acute leukemia. *Cancer Res.* 1987;47(24 Pt 1):6786–92.
20. Braess J, Wegendt C, Jahns-Streubel G, Kern W, Keye S, Unterhalt M, *et al.* Successful modulation of high-dose cytosine arabinoside metabolism in acute myeloid leukaemia by haematopoietic growth factors: no effect of ribonucleotide reductase inhibitors fludarabine and gemcitabine. *Br J Haematol.* 2000;109(2):388–95.
21. Lillemark JO, Plunkett W, Dixon DO. Relationship of 1-beta-D-arabinofuranosylcytosine in plasma to 1-beta-D-arabinofuranosylcytosine 5'-triphosphate levels in leukemic cells during treatment with high-dose 1-beta-D-arabinofuranosylcytosine. *Cancer Res.* 1985;45(11 Pt 2):5952–7.
22. Ossenkoppele GJ, Graveland WJ, Sonneveld P, Daenen SM, Biesma DH, Verdonck LF, *et al.* The value of fludarabine in addition to ARA-C and G-CSF in the treatment of patients with high-risk myelodysplastic syndromes and AML in elderly patients. *Blood.* 2004;103(8):2908–13.
23. Ozkaynak MF, Avramis VI, Carcich S, Ortega JA. Pharmacology of cytarabine given as a continuous infusion followed by mitoxantrone with and without amsacrine/etoposide as reinduction chemotherapy for relapsed or refractory pediatric acute myeloid leukemia. *Med Pediatr Oncol.* 1998;31(6):475–82.
24. Breithaupt H, Pralle H, Eckhardt T, von Hattingberg M, Schick J, Löffler H. Clinical results and pharmacokinetics of high-dose cytosine arabinoside (HD ARA-C). *Cancer.* 1982;50(7):1248–57.
25. Capizzi RL, Poole M, Cooper MR, Richards 2nd F, Stuart JJ, Jackson Jr DV, *et al.* Treatment of poor risk acute leukemia with sequential high-dose ARA-C and asparaginase. *Blood.* 1984;63(3):694–700.
26. Capizzi RL, White JC, Powell BL, Perrino F. Effect of dose on the pharmacokinetic and pharmacodynamic effects of cytarabine. *Semin Hematol.* 1991;28(3 Suppl 4):54–69.
27. Estey E, Plunkett W, Dixon D, Keating M, McCredie K, Freireich EJ. Variables predicting response to high dose cytosine arabinoside therapy in patients with refractory acute leukemia. *Leukemia.* 1987;1(8):580–3.
28. Qian SX, Li JY, Tian T, Shen YF, Jiang YQ, Lu H, *et al.* Effect of low-dose cytarabine and aclarubicin in combination with granulocyte colony-stimulating factor priming (CAG regimen) on the outcome of elderly patients with acute myeloid leukemia. *Leuk Res.* 2007;31(10):1383–8.
29. Willemze R, Zwaan FE, Colpin G, Keuning JJ. High dose cytosine arabinoside in the management of refractory acute leukaemia. *Scand J Haematol.* 1982;29(2):141–6.
30. Yamauchi T, Negoro E, Arai H, Ikegaya S, Takagi K, Takemura H, *et al.* Combined low-dose cytarabine, melphalan and mitoxantrone for older patients with acute myeloid leukemia or high-risk myelodysplastic syndrome. *Anticancer Res.* 2007;27(4C):2635–9.
31. Zhang WG, Wang FX, Chen YX, Cao XM, He AL, Liu J, *et al.* Combination chemotherapy with low-dose cytarabine, homoharringtonine, and granulocyte colony-stimulating factor priming in patients with relapsed or refractory acute myeloid leukemia. *Am J Hematol.* 2008;83(3):185–8.
32. Bauer W, Horvath Z, Hochtl T, Saiko P, Karl D, Fritzer-Szekeres M, *et al.* Amidox, an inhibitor of ribonucleotide reductase, potentiates the action of Ara-C in HL-60 human promyelocytic leukemia cells. *Nucleosides Nucleotides Nucleic Acids.* 2004;23(8–9):1541–4.
33. Fritzer-Szekeres M, Salamon A, Grusch M, Horvath Z, Hochtl T, Steinbrugger R, *et al.* Trimidox, an inhibitor of ribonucleotide reductase, synergistically enhances the inhibition of colony formation by Ara-C in HL-60 human promyelocytic leukemia cells. *Biochem Pharmacol.* 2002;64(3):481–5.
34. Plagemann PG, Behrens M, Abraham D. Metabolism and cytotoxicity of 5-azacytidine in cultured Novikoff rat hepatoma and P388 mouse leukemia cells and their enhancement by preincubation with pyrazofurin. *Cancer Res.* 1978;38(8):2458–66.
35. Gandhi V, Estey E, Keating MJ, Chucrallah A, Plunkett W. Chlorodeoxyadenosine and arabinosylcytosine in patients with acute myelogenous leukemia: pharmacokinetic, pharmacodynamic, and molecular interactions. *Blood.* 1996;87(1):256–64.
36. Gandhi V, Estey E, Plunkett W. Modulation of arabinosylcytosine metabolism during leukemia therapy. *Adv Exp Med Biol.* 1994;370:119–24.
37. Gandhi V, Nowak B, Keating MJ, Plunkett W. Modulation of arabinosylcytosine metabolism by arabinosyl-2-fluoroadenine in lymphocytes from patients with chronic lymphocytic leukemia: implications for combination therapy. *Blood.* 1989;74(6):2070–5.
38. Bhalla K, Swerdlow P, Grant S. Effects of thymidine and hydroxyurea on the metabolism and cytotoxicity of 1-B-D arabinofuranosylcytosine in highly resistant human leukemia cells. *Blood.* 1991;78(11):2937–44.
39. Colly LP, Richel DJ, Arentsen-Honders MW, Kester MG, ter Riet PM, Willemze R. Increase in Ara-C sensitivity in Ara-C sensitive and -resistant leukemia by stimulation of the salvage and inhibition of the *de novo* pathway. *Ann Hematol.* 1992;65(1):26–32.
40. Iversen N, Birkenes B, Torsdalen K, Djurovic S. Electroporation by nucleofector is the best nonviral transfection technique in human endothelial and smooth muscle cells. *Genet Vaccines Therapy.* 2005;3(1):2.
41. Cooper T, Ayres M, Nowak B, Gandhi V. Biochemical modulation of cytarabine triphosphate by clofarabine. *Cancer Chemother Pharmacol.* 2005;55(4):361–8.
42. Wang LM, White JC, Capizzi RL. The effect of Ara-C-induced inhibition of DNA synthesis on its cellular pharmacology. *Cancer Chemother Pharmacol.* 1990;25(6):418–24.
43. Verschuur AC, Van Gennip AH, Leen R, Voute PA, Brinkman J, Van Kuilenburg AB. Cyclopentenyl cytosine increases the phosphorylation and incorporation into DNA of 1-beta-D-arabinofuranosyl cytosine in a human T-lymphoblastic cell line. *Int J Cancer.* 2002;98(4):616–23.
44. Williams JC, Kizaki H, Weber G, Morris HP. Increased CTP synthetase activity in cancer cells. *Nature.* 1978;271(5640):71–3.
45. Cros E, Jordheim L, Dumontet C, Galmarini CM. Problems related to resistance to cytarabine in acute myeloid leukemia. *Leuk Lymphoma.* 2004;45(6):1123–32.
46. Schilsky RL, Ordway FS. Simultaneous determination of cytosine arabinoside, its nucleotides and metabolites by ion-pair high-performance liquid chromatography. *J Chromatogr.* 1985;337(1):63–71.
47. Plunkett W, Hug V, Keating MJ, Chubb S. Quantitation of 1-beta-D-arabinofuranosylcytosine 5'-triphosphate in the leukemic cells from bone marrow and peripheral blood of patients receiving 1-beta-D-arabinofuranosylcytosine therapy. *Cancer Res.* 1980;40(3):588–91.