Qingli Li, Mark J Lambrechts, Qiuyang Zhang, Sen Liu, Dongxia Ge, Rutie Yin, Mingrong Xi, Zongbing You.

Departments of Structural and Cellular Biology and Orthopaedic Surgery, Tulane Cancer Center and Louisiana Cancer Research Consortium, Tulane Center for Stem Cell Research and Regenerative Medicine, and Tulane Center for Aging, Tulane University Health Sciences Center, New Orleans, LA, USA; Department of Obstetrics and Gynecology, West China Second University Hospital, Sichuan University, Chengdu, People’s Republic of China.

Abstract: Glycine is a nonessential amino acid that is reversibly converted from serine intracellularly by serine hydroxymethyltransferase. Glyphosate and its degradation product, aminomethylphosphonic acid (AMPA), are analogs to glycine, thus they may inhibit serine hydroxymethyltransferase to decrease intracellular glycine synthesis. In this study, we found that glyphosate and AMPA inhibit cell growth in eight human cancer cell lines but not in two immortalized human normal prostatic epithelial cell lines. AMPA arrested C4-2B and PC-3 cancer cells in the G1/G0 phase and inhibited entry into the S phase of the cell cycle. AMPA also promoted apoptosis in C4-2B and PC-3 cancer cell lines. AMPA upregulated p53 and p21 protein levels as well as procaspase 9 protein levels in C4-2B cells, whereas it downregulated cyclin D3 protein levels. AMPA also activated caspase 3 and induced cleavage of poly (adenosine diphosphate [ADP]-ribose) polymerase. This study provides the first evidence that glyphosate and AMPA can inhibit proliferation and promote apoptosis of cancer cells but not normal cells, suggesting that they have potentials to be developed into a new anticancer therapy.

Keywords: serine hydroxymethyltransferase, prostate cancer, apoptosis

Introduction
Glycine is a nonessential amino acid that can be converted directly from serine in mammalian cells. Serine is derived from 3-phosphoglycerate. Serine hydroxymethyltransferase (SHMT) reversibly catalyzes the conversion of serine to glycine and vice versa. In the liver of vertebrates, glycine can be synthesized from N5,N10-methylene tetrahydrofolate by glycine synthase. Glycine is the precursor material for biosynthesis of protein, purine, and glutathione. In addition, glycine is converted into sarcosine (N-methylglycine) by glycine N-methyltransferase, and reversely, sarcosine can be converted into glycine by sarcosine dehydrogenase. The serum half-life of intravenously administered glycine varies from 1/2 hour to 4 hours. Recently, the importance of glycine in cancer cell biology has been revealed. In a panel of 60 human cancer cell lines that the National Cancer Institute uses for anticancer drug screening, rapidly proliferating cancer cells consumed extracellular glycine due to increased demand for glycine. In contrast, rapidly proliferating human normal cell lines released glycine. These findings suggest that targeting glycine consumption may become a new strategy in killing rapidly proliferating cancer cells, while not harming rapidly proliferating normal cells. Indeed, the use of short hairpin RNA (shRNA) to knockdown expression of SHMT2, thus blocking endogenous glycine synthesis,
effectively halted proliferation of LOX IMVI cells in the absence of extracellular glycine. In contrast, slowly proliferating cells were not affected by SHMT2 knockdown and deprivation of extracellular glycine.5

There are two isozymes of SHMT. SHMT1 encodes for the cytoplasmic and SHMT2 encodes for the mitochondrial isozyme.6,8 In mammalian cells, SHMT2 gene has an alternative promoter within intron 1, thus SHMT2 encodes for two transcripts, SHMT2 and SHMT2α.9 SHMT2 protein containing exon 1 (with mitochondrial-targeting sequence) is localized in mitochondria. SHMT2α protein without exon 1 is not imported into mitochondria efficiently and is localized predominantly in the cytoplasm and nucleus. SHMT1 protein, like SHMT2α, is also localized in the cytoplasm and nucleus, and both SHMT1 and SHMT2α catalyze production of one-carbon units from serine for nuclear de novo thymidylate biosynthesis.9 Interestingly, a glycine analog, aminomethylphosphonate (aminomethyl-phosphonic acid [AMPA]) (molecular formula CH3NO3P [Figure 1]), inhibits more than 95% of nuclear thymidylate biosynthesis that requires SHMT1 and SHMT2α, suggesting that AMPA is an effective inhibitor of SHMT1 and SHMT2α, as well as SHMT.9

AMPA is the primary degradation product of glyphosate (N-(phosphonomethyl)glycine; molecular formula C3H7NO3P [Figure 1]). Glyphosate is water soluble and chemically stable and is degraded by microbes to produce AMPA.10 Glyphosate is a weak organic acid consisting of a glycine moiety and a phosphonomethyl moiety. Glyphosate is a broad-spectrum herbicide that is used worldwide in agriculture, forestry, and aquatic weed control. It is applied to many crops in various commercial formulations. The major formulation is Roundup® (Monsanto Co, St Louis, MO, USA), in which glyphosate is formulated as the isopropylamine salt,11 whereas AMPA has no commercial use.10 In animal studies using [14C]glyphosate in rats, rabbits, and goats, approximately 30% of the oral dose was absorbed through the gastrointestinal tract. On day 7 after the oral dose of [14C]glyphosate in rats, the isotope was distributed throughout the animal body, with the highest concentration found in the bones. Almost all of the isotope was eliminated in urine and feces, with a very low level exhaled in air. The only metabolite was AMPA, which accounted for about 0.2% to 0.3% of the administered dose of glyphosate.10 In a study in rats, approximately 20% of the oral dose of AMPA was absorbed, which was excreted almost exclusively through the urine, with less than 0.1% of the dose expired as CO2.10 Glyphosate and AMPA have been found to present no significant toxicity in acute, subchronic, and chronic animal studies, nor any genotoxicity, teratogenicity, or carcinogenicity.10,12 In this study, we assessed the effects of glyphosate and AMPA on cancer cell growth. Our results suggest that glyphosate and AMPA inhibit cell growth in eight cancer cell lines but not in two immortalized human normal prostatic epithelial cell lines, at concentrations up to 50 mM.

Materials and methods
Cell culture

The immortalized human normal prostatic epithelial cell lines RWPE-1 and pRNS-1-1 were obtained from John Rhim (Uniformed Services University of the Health Sciences, Bethesda, MD, USA).13 Human castration-resistant prostate cancer cell line C4-2B was obtained from Leland WK Chung (Cedars-Sinai Medical Center, Los Angeles, CA, USA).14 Human prostate cancer cell lines (LNCaP, DU-145, and PC-3), human ovarian cancer cell lines (SKOV-3 and OVCAR-3), human cervical cancer HeLa cell line, and human lung cancer A549 cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). RWPE-1 and pRNS-1-1 cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) (Mediatech, Inc, Manassas, VA, USA) containing 10% fetal bovine serum (FBS) (HyClone Standard Fetal Bovine Serum; Thermo Fisher Scientific Inc, Waltham, MA, USA), 100 IU/mL penicillin/streptomycin, 5 μg/mL bovine insulin, 25 μg/mL bovine pituitary extract, and 6 ng/mL recombinant human epidermal growth factor (Sigma-Aldrich Corp, St Louis, MO, USA). LNCaP cells were cultured in

Figure 1 Chemical structure of glycine, AMPA, and glyphosate. Abbreviation: AMPA, aminomethylphosphonic acid.
T-Medium (Life Technologies Corp, Carlsbad, CA, USA) containing 5% FBS and 100 IU/mL penicillin/streptomycin. C4-2B and SKOV-3 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific) containing 10% FBS and 100 IU/mL penicillin/streptomycin. OVCAR-3 cells were cultured in RPMI-1640 medium containing 20% FBS, 100 IU/mL penicillin/streptomycin, 5 µg/mL bovine insulin, and 1 mM sodium pyruvate. DU-145, PC-3, HeLa, and A549 cells were cultured in DMEM medium containing 10% FBS and 100 IU/mL penicillin/streptomycin. DMEM contains 30 mg/L glucose and 42 mg/L L-serine; RPMI-1640 medium contains 10 mg/L glucose and 30 mg/L L-serine; and T-Medium contains 27 mg/L glucose and 37.8 mg/L L-serine. The cells were cultured in a 5% CO₂ humidified incubator at 37°C.

Cell viability assay
The live cell numbers were determined using the CellTiter-Glo® Luminous Cell Viability Assay (Promega Corp, Fitchburg, WI, USA). This assay is based on quantitation of the adenosine triphosphate (ATP), an indicator of metabolically active cells, which is a well-established method for cell proliferation and cytotoxicity assays.15-17 To optimize the experimental conditions, we performed pilot experiments and determined that there was a linear relationship ($r^2 = 0.98$) between the luminescent signal and the number of cells, from 1,000 to 40,000 cells per well. Therefore, we plated 4,000 cells per well, so that the cell number was less than 40,000 cells per well after 72 hours in culture. The cells were plated in 100 µL complete culture medium with FBS in Costar® opaque-walled 96-well plates (Thermo Fisher Scientific Inc). After overnight incubation, the cells were treated with glyphosate or AMPA (Sigma-Aldrich Corp) at final concentrations of 0, 15, 25, or 50 mM for 72 hours. Both glyphosate and AMPA were used in their native forms in all the experiments, and they were dissolved in complete medium with FBS prior to use. Each treatment group had triplicate wells. Wells containing the same medium without cells were used to assess the background luminescence. The amount of 100 µL CellTiter-Glo® Reagent was added into each well according to the instructions. Luminescence was read using a FLUOstar OPTIMA (BMG Labtech GmbH, Ortenberg, Germany) microplate reader. Cell viability was calculated as (luminescence of the treatment group – background luminescence) ÷ (luminescence of the control group – background luminescence) × 100%. The data are presented as the mean and standard error of the mean (SEM) of three independent experiments.

Cell apoptosis assay
C4-2B and PC-3 cells were plated in complete culture medium with FBS into 60 mm dishes. Sixteen hours later, the cells were treated without or with AMPA at a final concentration of 50 mM for 0, 24, 48, and 72 hours. The cells were trypsinized, washed once with PBS, pelleted, and resuspended in 70% ice-cold ethanol and stored at −20°C until the cell cycle analysis. The fixed cells were stained in a solution containing 0.1% Triton® X-100 (Sigma-Aldrich Corp), 0.2 mg/mL DNase-free RNase A, and 20 µg/mL propidium iodide for 30 min at room temperature in the dark. The percentages of cells at G1/G0, S, and G2/M phases were determined by flow cytometry analysis using DNA content frequency histogram deconvolution software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Western blot analysis
C4-2B cells were cultured in 60 mm dishes overnight. The cells were treated with AMPA at a final concentration of 50 mM for 0, 12, 24, 48, and 72 hours. Proteins were extracted from the treated cells in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM sodium fluoride, 0.5% Igepal® CA-630 [NP-40], 10 mM sodium phosphate, 150 mM sodium chloride, 25 mM Tris pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediaminetetraacetic acid [EDTA], 1.2 mM sodium vanadate) supplemented with protease inhibitor cocktail (Sigma-Aldrich Corp). An equal amount of proteins was subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in TBST buffer (25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween 20) for 2 hours and probed with the indicated primary antibodies overnight and then IRDye®680CW- or IRDye®680-conjugated secondary antibodies (LI-COR Biosciences Inc, Lincoln,
NE, USA) for 1 hour. The results were visualized using an Odyssey® Infrared Imager (LI-COR Biosciences Inc). For loading control, the membranes were stripped and probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The antibodies used were as follows: rabbit anti-poly (adenosine diphosphate [ADP]-ribose) polymerase (PARP), rabbit anti-caspase 3, rabbit anti-caspase 9, and mouse anti-cyclin D3 antibodies, purchased from Cell Signaling Technology Inc (Danvers, MA, USA); mouse anti-p21 and goat anti-p53 antibodies, obtained from Santa Cruz Biotechnology Inc (Dallas, TX, USA); and mouse anti-GAPDH antibodies, ordered from EMD Millipore Corp (Billerica, MA, USA).

Statistical analysis
All experiments were repeated three times, and the results represent mean ± SEM of three independent experiments. Statistical analysis was made using two-tailed Student’s t test. A P-value < 0.05 was considered statistically significant.

Results
Glyphosate inhibits cell growth in cancer cell lines but not in normal cell lines
Glyphosate, at concentrations of 15, 25, and 50 mM, did not significantly decrease the cell viability in the RWPE-1 and pRNS-1-1 cell lines compared with the untreated control group (P > 0.05) (Figure 2A and B). Glyphosate, at concentrations of 15 and 25 mM, did not decrease the cell viability in the LNCaP cell line; however, it decreased 27% of the cell viability at a concentration of 50 mM (P < 0.05) (Figure 2C). Glyphosate, at concentrations of 15, 25, and 50 mM, significantly decreased the cell viability in the C4-2B and DU-145 cell lines (P < 0.05 or P < 0.01) (Figure 2D and E), with a 73.4% and 39.3% decrease at the dose of 50 mM, respectively. Glyphosate, at a concentration of 15 mM, did not decrease the cell viability in the PC-3 and SKOV-3 cell lines; however, it significantly decreased the cell viability at concentrations of 25 and 50 mM (P < 0.05 or P < 0.01) (Figure 2F and G), with a 36.9% and 28% decrease at the dose of 50 mM in the PC-3 and SKOV-3 cell lines, respectively. Glyphosate, at concentrations of 15, 25, and 50 mM, significantly decreased the cell viability in the OVCAR-3 cell line (P < 0.05 or P < 0.01) (Figure 2H), with a 58.8% decrease at the dose of 50 mM. However, at a concentration of 50 mM, glyphosate only decreased about 25% and 17% of the cell viability in the HeLa and A549 cell lines, respectively, though the decrease was statistically significant (P < 0.05) (Figure 2I and J).

AMP A inhibits entry into the S phase of cell cycle and increases apoptosis
AMP A, at a concentration of 50 mM, significantly increased the number of C4-2B and PC-3 cells in the S phase of cell cycle, compared with the control group (P < 0.05 or P < 0.01) (Figure 4A and B). In contrast, AMP A significantly decreased the number of C4-2B and PC-3 cells in the S phase of cell cycle (P < 0.05 or P < 0.01), whereas the number of cells in the G2/M phase was not affected (Figure 4A and B). In addition, AMP A, at a concentration of 50 mM, significantly increased apoptosis of C4-2B and PC-3 cells in a time-dependent manner (P < 0.01) (Figure 4C and D).

AMP A induces changes in expression levels of genes involved in cell cycle and apoptosis
AMP A, at a concentration of 50 mM, increased the levels of cleaved PARP in the C4-2B cells in a time-dependent manner.
Figure 2 Glyphosate inhibits cell growth in cancer cell lines but not in normal cell lines.

Notes: (A–J) The cells were treated with 0, 15, 25, and 50 mM of glyphosate for 72 hours. Cell viability was determined using CellTiter-Glo® Luminescent Cell Viability Assay. Data represent the mean ± SEM obtained from three independent experiments. *P < 0.05 and **P < 0.01, compared with the untreated control group. Abbreviation: SEM, standard error of the mean.

Table 1 Half maximal inhibitory concentrations (IC_{50}) of glyphosate and AMPA in inhibition of the cell growth in the normal and cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>RWPE-1</th>
<th>pRNS-1-1</th>
<th>LNCaP</th>
<th>C4-2B</th>
<th>DU-145</th>
<th>PC-3</th>
<th>SKOV-3</th>
<th>OVCAR-3</th>
<th>HeLa</th>
<th>A549</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyphosate</td>
<td>109.1</td>
<td>372.8</td>
<td>90.4</td>
<td>34.2</td>
<td>63.9</td>
<td>63.2</td>
<td>89.0</td>
<td>42.7</td>
<td>89.4</td>
<td>136.7</td>
</tr>
<tr>
<td>AMPA</td>
<td>88.3</td>
<td>90.9</td>
<td>93.1</td>
<td>59.8</td>
<td>68.1</td>
<td>58.6</td>
<td>127.1</td>
<td>62.8</td>
<td>99.9</td>
<td>98.2</td>
</tr>
</tbody>
</table>

Note: Values are expressed in mM.

Abbreviation: AMPA, aminomethylphosphonic acid.
manner (Figure 5A). AMPA transiently increased the levels of p53 and its downstream gene p21, at 12 hours after treatment (Figure 5B). It also decreased cyclin D3 protein levels, starting from 12 hours after treatment (Figure 5B). Further, AMPA increased the levels of procaspase 9, starting from 24 hours after treatment (Figure 5B). In contrast, AMPA decreased the levels of procaspase 3, starting from 24 hours after treatment (Figure 5B).

**Discussion**

A previous study identified glycine as being consumed by rapidly proliferating cancer cell lines. Among the 60 human cancer cell lines tested in the previous study, five were included in the present study, namely, DU-145, PC-3, SKOV-3, OVCAR-3, and A549. Previously, it was shown that cancer cell proliferation was impaired by knocking down the expression levels of *SHMT2* that is responsible...
for intracellular glycine synthesis. Here we present data showing that glycine analogs, glyphosate and AMPA, inhibited cell growth in eight cancer cell lines, including four human prostate cancer cell lines (LNCaP, C4-2B, DU-145, and PC-3), two human ovarian cancer cell lines (SKOV-3 and OVCAR-3), one human cervical cancer cell line (HeLa), and one human lung cancer cell line (A549). In contrast, glyphosate and AMPA did not impair the growth of two normal cell lines (RWPE-1 and pRNS-1-1) at concentrations up to 50 mM. These findings suggest that glyphosate and AMPA can differentially affect cancer cell growth but not normal cell growth at concentrations up to 50 mM. However, at a higher concentration of 100 mM, AMPA decreased cell viability of the two normal cell lines, suggesting that potential adverse side effects may arise when the doses are too high. A safe therapeutic window may be limited to a drug concentration of between 50 mM to 100 mM. However, in clinical practice, it may be difficult to administer the drugs to reach this high concentration. Therefore, the use of glyphosate and AMPA in patients may be limited if the high IC\textsubscript{50} concentrations are difficult to achieve in patients’ blood. Nevertheless, glyphosate, particularly AMPA, may be a good lead compound for developing more potent inhibitors with low IC\textsubscript{50} concentrations.

Among the human cancer cell lines, the castration-resistant prostate cancer cell lines (C4-2B, DU-145, and PC-3) and ovarian cancer cell line (OVCAR-3) appear to be...
more sensitive to glyphosate and AMPA than are the other human cancer cell lines. The IC\textsubscript{50} concentrations varied from 34.2 to 68.1 mM among the four sensitive cancer cell lines (C4-2B, DU-145, PC-3, and OVCAR-3). In contrast, the IC\textsubscript{50} concentrations were between 89 and 136.7 mM in other cancer cell lines, including LNCaP, SKOV-3, HeLa, and A549 (Table 1), suggesting that these four cell lines are resistant to glyphosate and AMPA. The differences in sensitivity may be caused by many factors that are not clearly understood because these cell lines are derived from different genetic backgrounds. The rate of cell proliferation may be one of the factors, as suggested by previous study.\textsuperscript{5} Indeed, the C4-2B cells were more sensitive to glyphosate and AMPA than LNCaP cells. Coincidentally, LNCaP cells grow slower than C4-2B cells, which are castration-resistant cells derived from the hormone-sensitive LNCaP cells.\textsuperscript{14} This observation indicates that glyphosate and AMPA are more effective in inhibiting growth of rapidly proliferating cancer cells.

Cell growth in a population of cells represents the net outcome of proliferation and apoptosis. Our data indicate that AMPA can arrest cancer cells in the G1/G0 phase of cell cycle, thus inhibiting entry into the S phase. On the other hand, AMPA can enhance apoptosis of cancer cells, as shown by the increased rates of annexin-V-positive cells and increased levels of cleaved PARP, an indicator of apoptosis. Therefore, AMPA inhibits cancer cell growth through inhibition of cellular proliferation and promotion of apoptosis. The molecular mechanism may be that AMPA upregulates the p53 protein level, which subsequently increases p21 protein level. Activation of the p53-p21 pathway is known to cause G1-phase arrest and apoptosis in mammalian cells.\textsuperscript{20–22} AMPA downregulates the expression of cyclin D3, which may also contribute to the cell cycle arrest.\textsuperscript{23} AMPA increases procaspase 9 levels and simultaneously decreases procaspase 3 levels, which may mediate apoptosis, as shown in a previous study.\textsuperscript{24} However, how AMPA initiates these molecular changes and whether these changes apply to other cancer cell lines remain to be determined.

To our best knowledge, this is the first study showing that glycine analogs can inhibit proliferation and promote apoptosis of cancer cells but not normal cells, in vitro at concentrations up to 50 mM. Higher concentrations of the chemicals may affect normal cells, thus producing adverse side effects. These findings suggest that animal studies are warranted to assess the efficacy of glyphosate and AMPA in the treatment of tumors growing in animals and to test whether the effective inhibitory concentrations can be achieved in animal blood. If a positive outcome is obtained in preclinical animal study, it will be feasible to conduct human clinical trials because glyphosate and AMPA are of little toxicity to animals and humans.\textsuperscript{10} On the other hand, more potent inhibitors may be developed using glyphosate and AMPA as lead compounds. Based on our findings and the previous report,\textsuperscript{3} it appears promising to develop a new anticancer therapy targeting glycine metabolism.

**Acknowledgments**

This work was partly supported by a grant from the Department of Defense (PC121647), two grants from the National Institute of General Medical Sciences (P20GM103518) and the National Cancer Institute (R01CA174714) of the National Institutes of Health, the Developmental Fund of Tulane Cancer Center (TCC), and the Louisiana Cancer Research Consortium (LCRC) Fund (to ZY). Dr Zongbing You conceived the idea and designed the experiments. The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The TCC and LCRC FACS Core Facilities were used to conduct this study. Dr Qingli Li received a scholarship from the State Scholarship Fund via China Scholarship Council (CSC) for her training under Dr Zongbing You at Tulane University.
Disclosure

The authors report no conflicts of interest in this work.

References