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Aldo-keto reductases-mediated cytotoxicity of 2-deoxyglucose: A novel anticancer mechanism

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2-Deoxyglucose (2DG) is a non-metabolizable glucose analog currently in clinical trials to determine its efficacy in enhancing the therapeutic effects of radiotherapy and chemotherapy of several types of cancers. It is thought to preferentially kill cancer cells by inhibiting glycolysis because cancer cells are more dependent on glycolysis for their energy needs than normal cells. However, we found that the toxicity of 2DG in cancer cells is mediated by the enzymatic activities of AKR1B1 and/or AKR1B10 (AKR1Bs), which are often overexpressed in cancer cells. Our results show that 2DG kills cancer cells because, in the process of being reduced by AKR1Bs, depletion of their cofactor NADPH leads to the depletion of glutathione (GSH) and cell death. Furthermore, we showed that compounds that are better substrates for AKR1Bs than 2DG are more effective than 2DG in killing cancer cells that overexpressed these 2 enzymes. As cancer cells can be induced to overexpress AKR1Bs, the anticancer mechanism we identified can be applied to treat a large variety of cancers. This should greatly facilitate the development of novel anticancer drugs.

KEYWORDS
2-deoxyglucose, AKR1B1, AKR1B10, glutathione (GSH), oxidative stress

1 | INTRODUCTION

2-Deoxyglucose (2DG) is a mild anticancer drug that is being tested in clinical trials to determine its efficacy to augment chemotherapy and radiotherapy. The cytotoxicity of 2DG is not as severe as some of the anticancer drugs. However, its specificity in killing cancer cells makes it a good candidate to enhance the efficacy of other cancer therapies. It has been shown to have synergistic effects in enhancing the killing of breast cancer cells by 5-fluorouracil, cisplatin, and cyclophosphamide.[1] It enhances apoptosis of melanoma cells induced by tumor necrosis factor (TNF)-related apoptosis ligands,[2] and also enhances the apoptotic effect of several histone deacetylase inhibitors on breast, glioma and cervical cancer cells.[3] 2DG, being a non-metabolizable glucose analog, is thought to preferentially kill cancer cells by inhibiting glucose metabolism because cancer cells are more dependent on glycolysis for their energy needs than normal cells.[4,5] However, 2DG reduces glycolysis to about 15%-40% of normal cells only, not enough to cause glucose starvation.[6] More importantly, it has been shown that under normoxic conditions, 2-fluorodeoxy-D-glucose (2FDG), a more potent glycolysis inhibitor than 2DG, is less effective than 2DG in killing cancer cells.[7] These observations suggest that under normoxic conditions, 2DG does not kill cancer cells by inhibition of glucose metabolism. Even though the mechanism is not clear, the cancer-specific cytotoxicity of 2DG is intriguing. A better understanding of its mechanism might lead to the development of better anticancer drugs.

In our earlier experiments, we found that 2DG-induced oxidative stress in cardiomyocytes was attenuated by treatment with an AKR1B1 inhibitor.[8] AKR1B1, or aldose reductase, was first

Zhang and Ken Yung contributed equally to this work.

recognized as 1 of the major contributors to diabetic complications.[9,10] It reduces glucose, which is elevated in diabetic patients, to sorbitol. In the process, its cofactor NADPH is depleted. NADPH is also the cofactor for glutathione reductase (GR), an enzyme that regenerates GSH from reduced GSH (GSSH).[11] Thus, under hyperglycemic conditions, AKR1B1 activity depletes NADPH, depriving GR of using this cofactor to regenerate GSH. This causes oxidative stress because GSH is 1 of the major cellular antioxidants.

2DG is 1 of the broad range of substrates for AKR1B1 and a closely related enzyme AKR1B10, also known as aldose reductase-like enzyme or ARL-1.[12] These 2 enzymes are members of the AKR1B subgroup of the family of aldo-keto reductases. Of their amino acid sequences, 71.4% are identical to each other. They also have similar substrate specificity.[12] AKR1B1/AKR1B10 (AKR1Bs) are often overexpressed in various cancers.[13] This is most likely because 1 of their transcription factors, the nuclear factor erythroid 2-related factor 2 (Nrf2), is often constitutively activated in cancer cells.[14] These 2 enzymes are effective in neutralizing toxic metabolites such as methylglyoxal and 4-hydroxynonenal.[15,16] As cancer cells with a higher metabolic rate might produce more of these toxic aldehydes, overexpression of these enzymes might promote cancer cell survival.[17] Therefore, inhibition of AKR1Bs has been suggested as a means to stop cancer growth.[18]

We, however, propose to make use of the activities of AKR1Bs to specifically kill cancer cells that overexpress these enzymes. In the presence of their preferred substrates, the reduction activity of AKR1Bs would deplete NADPH, leading to the depletion of GSH, increased oxidative stress, and cell death. This report presents the experimental support of this hypothesis.

2 | MATERIALS AND METHODS

2.1 | Cell culture

SKOV3, HCT116, CaCo2 and HT29 cells were purchased from ATCC (Manassas, VA, USA). HepG2 and SW480 cells were kindly provided by Prof. ZhiLing Yu (Hong Kong Baptist University, Hong Kong). SW480 cells were cultured in 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA), HT29 cells in McCoy's 5A medium (Thermo Fisher Scientific), and other cells in DMEM medium (Thermo Fisher Scientific). All cell culture medium was supplemented with 10% FBS (Thermo Fisher Scientific), 1% Penicillin-Streptomycin-Neomycin (Thermo Fisher Scientific), and cultured in a humidified incubator containing 5% CO_2 at 37°C.

2.2 | Transfection of AKR1B1/10 siRNA

Cancer cells were seeded on a culture plate and grown to 60% confluence, and then transfected with AKR1B1-siRNA (sc-37119; Santa Cruz Biotechnology, Dallas, TX, USA) and/or AKR1B10-siRNA (sc-72341; Santa Cruz Biotechnology); scrambled negative control RNA was used as control (siNC). AKR1Bs siRNA was transfected into the cells using Lipofectamine™ 3000 (Thermo Fisher Scientific) in Opti-MEM medium (Thermo Fisher Scientific) according to the manufacturer's instructions. Cancer cells were cultured for at least 48 hours after siRNA transfection before further tests were carried out. Silencing of AKR1B1/ARK1B10 was confirmed by western blot, and cytotoxicity assay was carried out as described below.

2.3 | Cytotoxicity assay

Cytotoxicity was assessed by cell survival rate using MTT assay. After cells were treated with substrates (2DG, glyceraldehyde and diacetyl; Sigma, St Louis, MO, USA) for the length of time indicated, MTT (Sigma) was added and incubated for another 4 hours. DMSO (Sigma) was then added to dissolve the formazan product, and the absorbance of dissolved dye was measured at 540 nm using an automatic microplate reader.

2.4 | Western blot

Proteins were extracted in protein extraction reagent (Novagen, Madison, WI, USA) with protease inhibitor cocktail (Calbiochem, San Diego, CA, USA). Protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Total protein (30 μg) per sample was separated using SDS-PAGE and electrotransferred to PVDF membranes (Bio-Rad). Membranes were then soaked in 5% non-fat milk for 1 hour and then incubated with anti-AKR1B10 antibody (1:3000; Abcam, Cambridge, MA, USA) or anti-AKR1B1 antibody (1:3000) kindly provided by Dr Deliang Cao (Southern Illinois Medical School, Springfield, IL, USA), then incubated with secondary antibodies. Signals were visualized with detection kit (AbFrontier, Seoul, Korea) and then exposed with ChemiDoc Touch imaging system (Bio-Rad) or the X-Omat LS film (Sigma) in a dark room. β-Actin protein levels were measured as a reference for equivalence of protein loading.

2.5 | GSH and γ-glutamylcysteine ligase activity assay

Scraped cells were disrupted by ultrasonicator on ice followed by centrifugation at 1200 *g* at 4°C. The supernatant was used for GSH assay using a GSH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), concentrations of GSH in cell lysates were determined using the standard GSH calibration curve. γ-Glutamylcysteine ligase (GCL) activity assay was carried out using a commercial kit (Nanjing Jiancheng Bioengineering Institute).

2.6 | AKR1Bs activity assay

Activity assay of AKR1Bs was conducted in 1 mL of the reaction mixture containing 135 mmol/L sodium phosphate buffer (pH 6.2 for AKR1B1 or pH 7.0 for AKR1B10), 0.2 mmol/L NADPH (Sigma), 0.3 mol/L ammonium sulfate, 2 μg purified proteins (Abcam) and 20 mmol/L of their substrate as indicated. The reaction mixture was incubated at 30°C for 30 minutes. Decrease of NADPH was

monitored by spectrophotometer at 340 nm. Enzyme activity was calculated as the amount of NADPH oxidized/min per mg protein.[19]

2.7 | Acute toxicity test

ICR mice (18-22 g) were acquired from the Laboratory Animal Services Centre, The Chinese University of Hong Kong (Hong Kong). There were 4 males and 4 females in each group. Diacetyl or glyceraldehyde was dissolved in saline and given to the mice by a single iv injection with the amount as indicated. Mice were observed daily for 14 days. Mortality was recorded for the calculation of median lethal dose (LD_{50}). All animals were killed at the end of the experiment. The protocol was approved by Department of Health, the Government of HKSRA (Ref.: (15-34) in DH/HA&P/8/2/6 Pt4).

2.8 | Tumor xenograft studies

Six-week-old male BALB/C nude mice were purchased from the Laboratory Animal Unit of the University of Hong Kong and raised in a specific pathogen-free (SPF) room. Human HepG2 tumor xenografts were established by injecting 5×10^6 HepG2 cells/mice in the right flanks of the nude mice. Treatment was initiated when the tumor grew to approximately 200 ± 100 mm^3. Mice were randomized and allocated to different groups (6 mice per group). They received daily tail vein injection for 3 weeks of 1 of the following: glyceraldehyde (500 mg/kg); diacetyl (80 mg/kg); normal saline. Mice were weighed and tumors sizes were measured with calipers every 3 days. Tumor volumes were calculated with the formula: length $\times$ width^2/2. Animals were humanely killed at the end of the experiment and their tumors were weighed. The protocol was approved by Department of Health, the Government of HKSRA (Ref.: (15-31) in DH/HA&P/8/2/6 Pt4).

2.9 | Statistical analysis

Quantitative results are expressed as mean $\pm$ SD. Data were analyzed by one-way analysis of variance. Statistical significance was defined as $P < .05$. The analysis was conducted using GraphPad Prism.

3 | RESULTS

3.1 | Cells with higher levels of AKR1Bs were more sensitive to 2DG

To determine the relationship between sensitivity to 2DG toxicity and cellular levels of AKR1Bs, several cancer cell lines were examined to determine their sensitivity to 2DG and their expression levels of these 2 enzymes. HT29 and SW480 were more resistant to 2DG whereas HepG2, SKOV3, HCT116, and CaCo2 were more sensitive (Figure 1A; Figure S1). Western blot analysis showed that the resistant cells, HT29 and SW480, had lower levels of AKR1B1 and AKR1B10, and 2DG-sensitive cells HCT116 and CaCo2 had high levels of AKR1B1. The other 2DG-sensitive cells, HepG2 and SKOV3, had high levels of both AKR1B1 and AKR1B10 (Figure 1B). To further confirm our hypotheses, expression of AKR1B1 and AKR1B10 in HepG2 and SKOV3 cells (Figure 1C) and AKR1B1 in HCT116 and CaCo2 cells (Figure 1D) was suppressed by RNA interference. Silencing AKR1Bs had no obvious effect on cell viability but significantly attenuated the cytotoxicity of 2DG in these cancer cells (Figure 1E). Thus, sensitivity to 2DG toxicity is directly correlated with the cellular levels of AKR1Bs. However, it should be pointed out that, compared with HepG2 and SKOV3, CaCo2 and HCT116 appeared to have lower levels of AKR1B1 and very little AKR1B10 and yet they were more sensitive to 2DG. It is possible that these 2 cell lines have lower capacity for de novo synthesis of GSH or have a higher rate of GSH efflux such that small decreases in GSH regeneration would lead to severe depletion of GSH.

3.2 | Inhibition of AKR1Bs protects cells against the toxic effects of 2DG

To confirm that 2DG cytotoxicity was a result of the enzymatic activities of AKR1Bs, the effect of AKR1Bs inhibitors on cell survival in the presence of 2DG was examined. We used 2 types of AKR1Bs inhibitors, tolrestat (Biochempartner, Shanghai, China) and fidarestat, kindly provided by Prof. SK Chung (The University of Hong Kong, Hong Kong). Tolrestat is able to inhibit both AKR1B1 and AKR1B10, whereas fidarestat can inhibit AKR1B1 only.[20] The concentrations of tolrestat and fidarestat used had no conspicuous effect on cell survival. Tolrestat dose-dependently protected all 4 cell lines against 2DG toxicity, whereas fidarestat protected HCT116 and CaCo2 cells, but was less effective in protecting HepG2 and SKOV3 cells (Figure 2A-D). This is most likely a result the fact that HepG2 and SKOV3 overexpress both AKR1B1 and AKR1B10, and AKR1B10 remained active in the presence of fidarestat.

3.3 | GSH level in 2DG-treated cells was restored by AKR1Bs inhibitor

Earlier reports have established that 2DG toxicity in cancer cells is associated with depletion of GSH leading to oxidative stress, although the mechanism leading to GSH depletion is not entirely clear.[21,22] If the hypothesis that 2DG toxicity in cancer cells is mediated by AKR1Bs activities is correct, inhibiting the activities of these 2 enzymes should attenuate the 2DG-induced depletion of GSH. As shown in Figure 2E,F, the GSH levels in all 4 cell lines that are sensitive to 2DG (HepG2, SKOV3, HCT116 and CaCo2) decreased significantly in the presence of 2DG. Tolrestat by itself did not affect the GSH level but significantly restored the GSH levels in all 4 cell lines treated with 2DG. Fidarestat alone did not affect the GSH level, but was able to increase the levels of GSH in HCT116 and CaCo2 cells, but not in HepG2 and SKOV3 cells, presumably because it was not able to inhibit AKR1B10 in these 2 cell lines. Furthermore, the activity of GCL, responsible for the synthesis of GSH, was not affected by fidarestat and tolrestat (Figure S2). Taken together, these observations indicated that the AKR1Bs inhibitors increased GSH levels in 2DG-induced cancer cells was a result their inhibition of AKR1Bs.

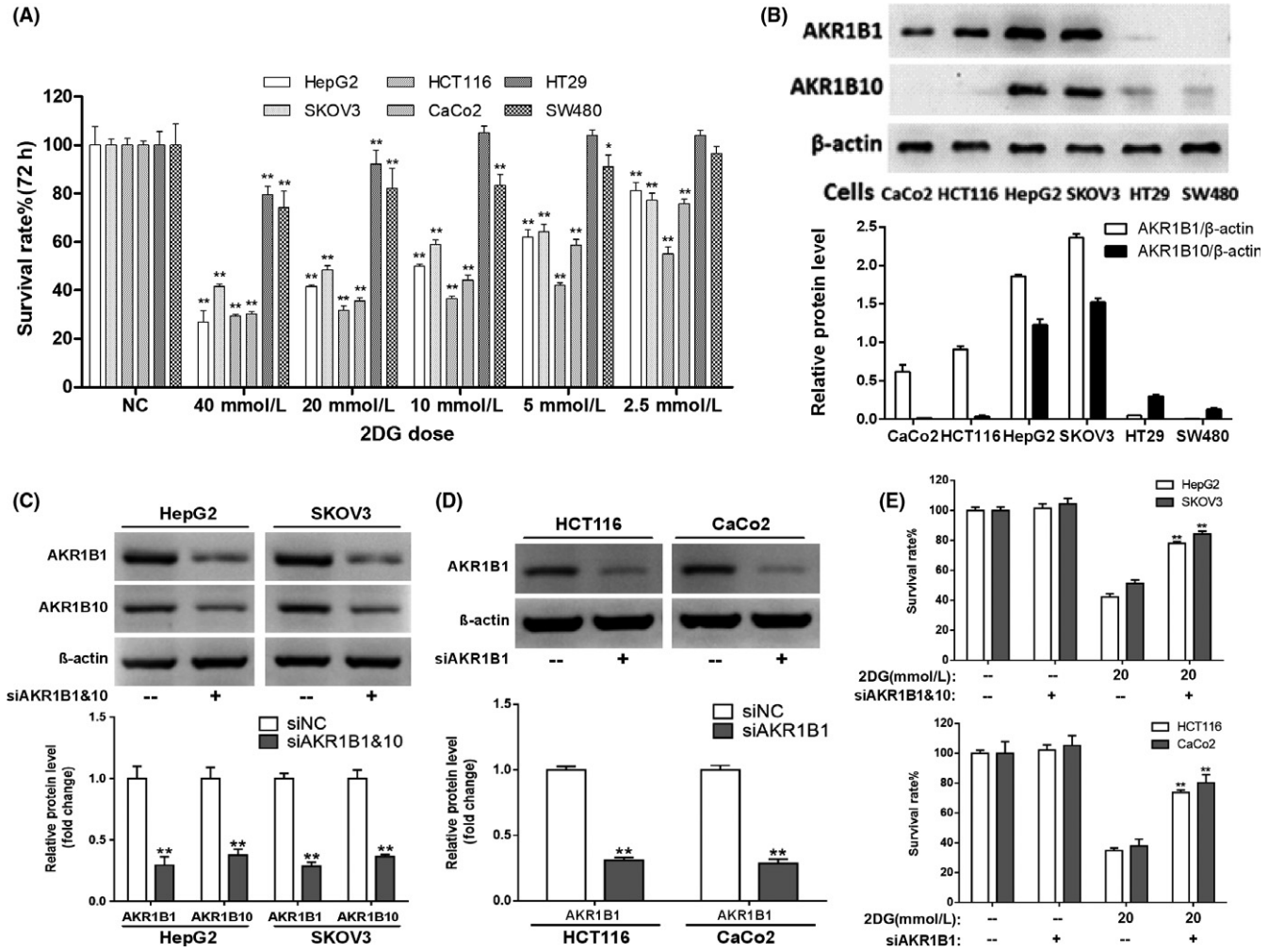

FIGURE 1 Sensitivity of cells to 2-deoxyglucose (2DG) is correlated with their AKR1B1 and/or AKR1B10 (AKR1Bs) protein levels. A, Survival rate of cancer cells treated with 2DG for 72 h. $^{**}P < .01$ vs NC group. Bars represent mean ± SD (n = 5). B, Comparison of expression levels of AKR1B1 and AKR1B10 in 6 types of cancer cell and quantitative analysis of band intensity relative to β-actin. Bars represent mean ± SD (n = 3). Both AKR1B1 and AKR1B10 protein correspond to a 36-kDa band. Bottom band shows the same membrane re-probed for β-actin, which corresponds to a 42-kDa band. C,D, Silencing of AKR1B1/10 after siRNA transfection in HepG2 and SKOV3 cells (C), and silencing of AKR1B1 in HCT116 and CaCo2 cells (D). Quantitative analysis of band intensity relative to β-actin. Bars represent mean ± SD (n = 3), $^{**}P < .01$ vs control group. E, Viability of transfected cell induced by 2DG was measured after transfection with siRNA for 48 h using MTT assay. $^{**}P < .01$ vs 2DG group only. Bars represent mean ± SD (n = 5). NC, normal control

3.4 | Increasing cellular levels of AKR1Bs rendered cells more sensitive to 2DG

To further confirm that sensitivity to 2DG toxicity is a result of overexpression of AKR1Bs, the 2 cell lines (HT29 and SW480) that had low levels of these 2 enzymes were treated with MG-132 (Sigma) and bortezomib (BioVision, Milpitas, CA, USA) before determining their sensitivity to 2DG. These 2 compounds were previously shown to be able to increase the expression levels of AKR1B1 and AKR1B10,[23] and here we show that they did not affect the enzyme activity of AKR1B1 and AKR1B10. When cells were treated with MG-132 or bortezomib alone, there was no effect on cell viability (Figure 3A). Pretreatment with MG-132 or bortezomib for 24 hours before the addition of 2DG led to significantly more cell death (Figure 3B,C). MG-132 was able to increase the protein levels of AKR1B1 and AKR1B10 in HT29 and SW480 cells (Figure 3D,E). Bortezomib, however, increased AKR1B1 and AKR1B10 protein levels in HT29 cells, but, in SW480 cells, it induced AKR1B1 only (Figure 3F,G). These results are similar to those reported earlier.[23] These findings indicated that increased levels of AKR1Bs made the cells more sensitive to this drug.

3.5 | Cells that overexpress AKR1Bs are sensitive to other substrates of these 2 enzymes

To determine whether the mechanism of AKR1Bs-mediated toxicity of 2DG is applicable to other substrates of these 2 enzymes, we tested 2 other substrates of AKR1Bs, glyceraldehyde and diacetyl.[12] Both glyceraldehyde and diacetyl dose dependently and

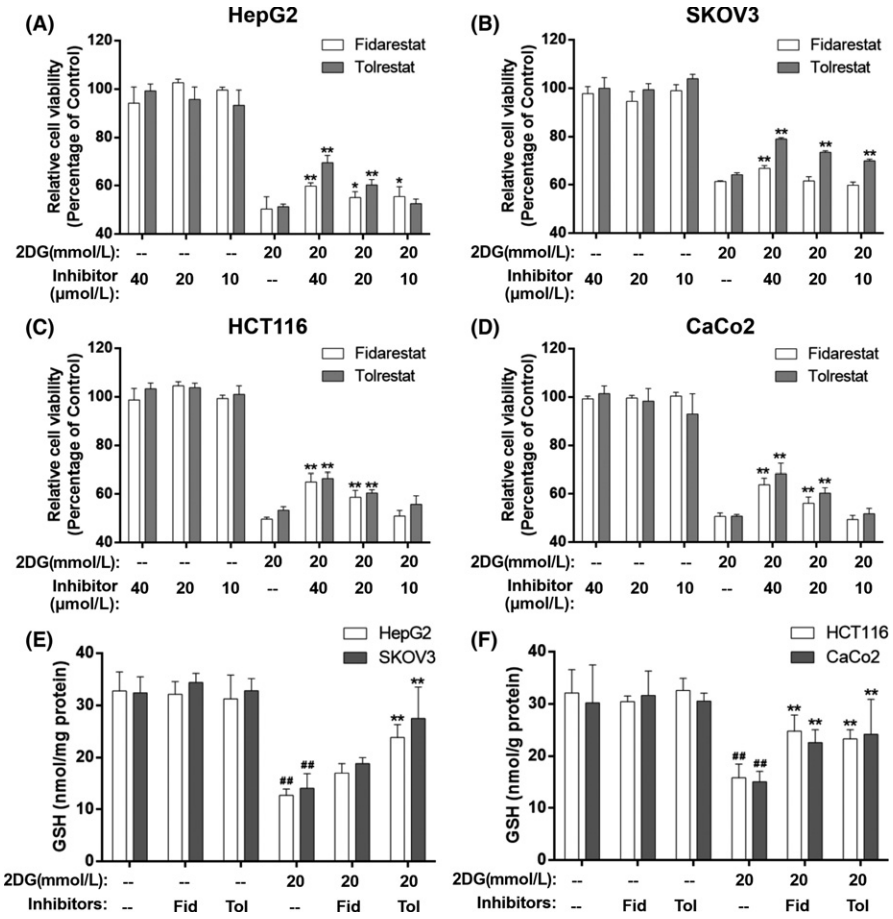

FIGURE 2 AKR1B1 and/or AKR1B10 (AKR1Bs) inhibitors protect cancer cells against 2-deoxyglucose (2DG) toxicity. A-D, Survival rate of 4 types of cancer cell after treatment with the inhibitors for 24 h, followed by the addition of 2DG (20 mmol/L) for an additional 48 h. A, HepG2 cells. B, SKOV3 cells. C, HCT116 cells. D, CaCo2 cells. $^{*}P < .05$; $^{**}P < .01$ vs 2DG induced cells only. E, Glutathione (GSH) level is restored by AKR1Bs inhibitor (40 μmol/L for 24 h, followed by the addition of 2DG for 48 h) in 2DG-treated HepG2 and SKOV3 cells. F, GSH level is restored by AKR1Bs inhibitor in 2DG-treated HCT116 and CaCo2 cells. $^{\#\#}P < .01$ vs NC group; $^{**}P < .01$ vs 2DG group. Bars represent mean ± SD (n = 5). NC, normal control

preferentially killed cells that have high levels of AKR1Bs (HepG2, SKOV3, HCT116 and CaCo2; Figure 4A,B; Figure S3). Cells (HT29 and SW480) that have low levels of these 2 enzymes were more resistant to these 2 substrates.

3.6 | Cytotoxicity of glyceraldehyde and diacetyl in AKR1Bs-overexpressing cells is also a result of depletion of GSH

Central to our proposed mechanism of AKR1Bs-mediated cytotoxicity is the depletion of GSH as a consequence of the activities of these enzymes. Thus, GSH levels in cells treated with glyceraldehyde and diacetyl were determined. As shown in Figure 4C, glyceraldehyde and diacetyl significantly reduced GSH levels in cells that overexpress AKR1Bs (HepG2, SKOV3, HCT116 and CaCo2). Cells with low levels of AKR1Bs (HT29 and SW480) showed modest reduction of GSH when treated with glyceraldehyde or diacetyl. This result indicates that the mechanism of cytotoxicity of 2DG, glyceraldehyde, and diacetyl is the same: the depletion of GSH as a consequence of increased AKR1Bs activities.

3.7 | Cytotoxicity of substrates of AKR1Bs depends on how efficiently they are being reduced by these enzymes

The proposed model of AKR1Bs-mediated cytotoxicity predicts that the better the substrate for these enzymes, the more potent is its cytotoxicity. We therefore compared the catalytic efficiency of AKR1Bs for 2DG, glyceraldehyde and diacetyl. These 3 compounds were used as substrates in standard AKR1B1 and AKR1B10 enzyme assays. As shown in Figure 5A, for AKR1B1, glyceraldehyde was the best substrate, followed by diacetyl, then 2DG; for AKR1B10, diacetyl was the best substrate, followed by glyceraldehyde, then 2DG. Data shown in previous results already showed that glyceraldehyde is the most toxic to the cells, followed by diacetyl, then 2DG. To ease comparison, percentages of cells that survived after the treatment with these 3 compounds from Figure 4 and Figure S1 were redrawn in Figure 5B,C.

3.8 | Inhibition of GSH synthesis enhanced the toxicity of AKR1Bs substrates and increasing the levels of GSH protected cells against their toxicity

Our hypothesis states that the toxicity of the substrates of AKR1Bs is a result of the depletion of GSH. This was investigated using glyceraldehyde and diacetyl as their substrates. DL-Buthionine-sulfoximine (BSO; Sigma) is a drug that reversibly inhibits glutamate cysteine ligase activity,[24] and consequently inhibits GSH synthesis, resulting in GSH depletion. The presence of BSO enhanced the toxicity of both glyceraldehyde (Figure 5D) and diacetyl (Figure 5E) in all 4 AKR1Bs-overexpressing cells. *N*-Acetyl-cysteine (NAC; Sigma), a precursor of GSH synthesis, was used to increase the cellular concentrations of GSH.[25] As shown in Figure 5F,G, NAC was able to protect the cells against the toxicity of glyceraldehyde and diacetyl

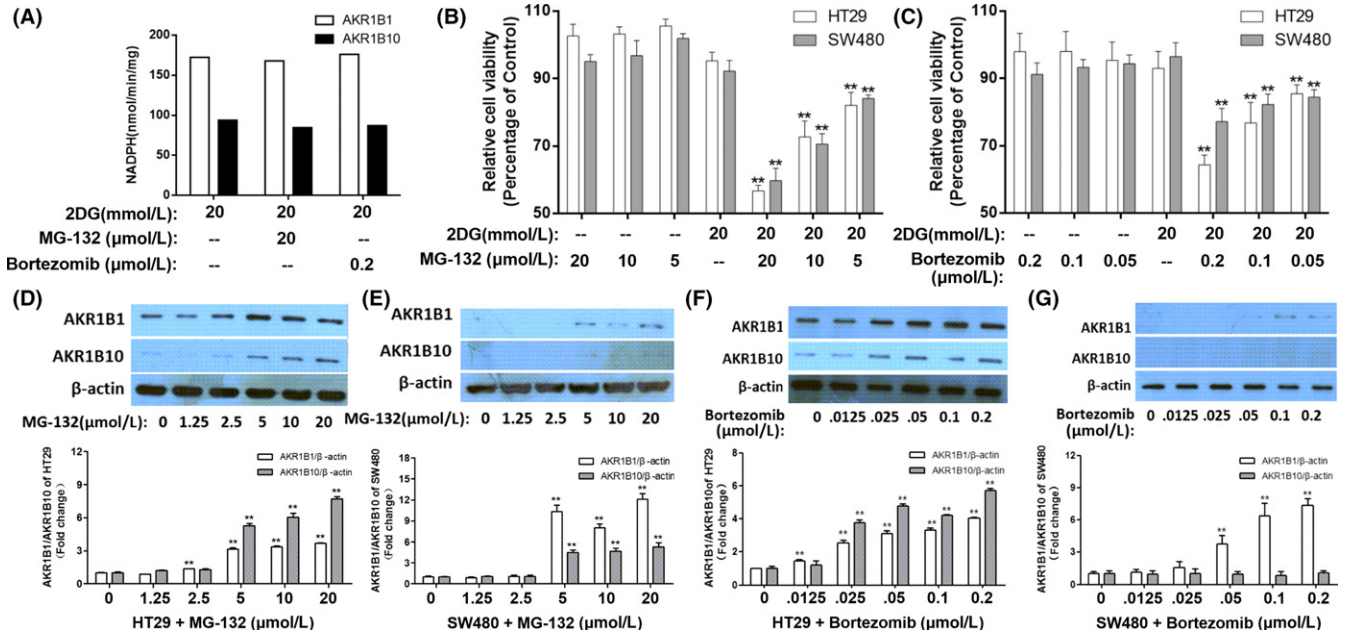

FIGURE 3 Increasing the cellular levels of AKR1B1 and/or AKR1B10 (AKR1Bs) made the cells more sensitive to 2-deoxyglucose (2DG). A, Effect of MG-132 and bortezomib on AKR1B1s activity, expressed as the amount of NADPH oxidized/min per mg protein. B,C, Cells were treated with MG-132 or bortezomib for 24 h before the addition of 2DG. $^{**}P < .01$ vs 2DG group. Bars represent mean ± SD (n = 5). B, Sensitivity to 2DG of MG-132-treated cells. C, Sensitivity to 2DG of bortezomib-treated cells. D-G, Western blot analysis of cells treated with MG-132 or bortezomib. $^{**}P < .01$ vs 0-dose group. Bars represent mean ± SD (n = 3). D, MG-132-treated HT-29 cells. E, MG-132-treated SW480 cells. F, Bortezomib-treated HT-29 cells. G, Bortezomib-treated SW480 cells

in all 4 cell lines. Taken together, these results strongly support the hypothesis that the substrates of AKR1Bs kill cancer cells as a result of the depletion of their cofactor NADPH, leading to the depletion of GSH.

3.9 | Acute toxicity of diacetyl and glyceraldehyde in mice

To prepare for the study of the antitumor effects of diacetyl and glyceraldehyde, the toxicity of these compounds in mice was examined. These compounds were injected into ICR mice with the amounts indicated in Table 1. From the results shown in Table 1, LD_{50} of diacetyl was estimated to be 410 mg/kg body weight. For glyceraldehyde, LD_{50} was found to be >2000 mg/kg body weight. Body weight of ICR mice increased normally in both diacetyl and glyceraldehyde groups (Figure S4). Because of solubility problems, higher amounts of glyceraldehyde could not be tested.

3.10 | Substrates of AKR1Bs inhibit tumor growth in a xenograft model

To determine the antitumor effects of AKR1Bs substrates in vivo, glyceraldehyde (500 mg/kg) or diacetyl (80 mg/kg) or normal saline were injected into tumor xenograft mice by tail veins daily for 21 days. Tumor size and body weight were measured every 3 days. The antitumor effect of glyceraldehyde is shown in Figure 6A-D. Glyceraldehyde did not affect the body weight of the mice (Figure 6B). Treatment with glyceraldehyde significantly reduced tumor volume (Figure 6C) and tumor weight (Figure 6D). The antitumor effect of diacetyl is shown in Figure 6E-H. Similarly, diacetyl did not affect the body weight of the mice (Figure 6F). Treatment with diacetyl also significantly reduced tumor volume (Figure 6G) and tumor weight (Figure 6H) in nude mice. These results strongly support our hypothesis that substrates of AKR1Bs can be used as anticancer drugs.

4 | DISCUSSION

In the present study, we showed that the cancer-specific cytotoxicity of 2DG is a result of the depletion of GSH mediated by the activities of AKR1Bs rather than through its inhibition of glycolysis. This is supported by several lines of evidence as follows.

1. Sensitivity to 2DG toxicity is correlated with the level of expression of AKR1Bs.
2. Suppression of AKR1Bs expression or inhibition of AKR1Bs activities protected the cells against 2DG toxicity. This is not a nonspecific effect of the inhibitors because fidarestat, which inhibits AKR1B1 only, was ineffective in protecting cells that overexpress both AKR1B1 and AKR1B10.
3. Increasing the cellular levels of AK1RBs rendered the cells more sensitive to 2DG.
4. 2DG-induced depletion of GSH was attenuated by AKR1Bs inhibitors, and these inhibitors had no effect on the activity of GCL.

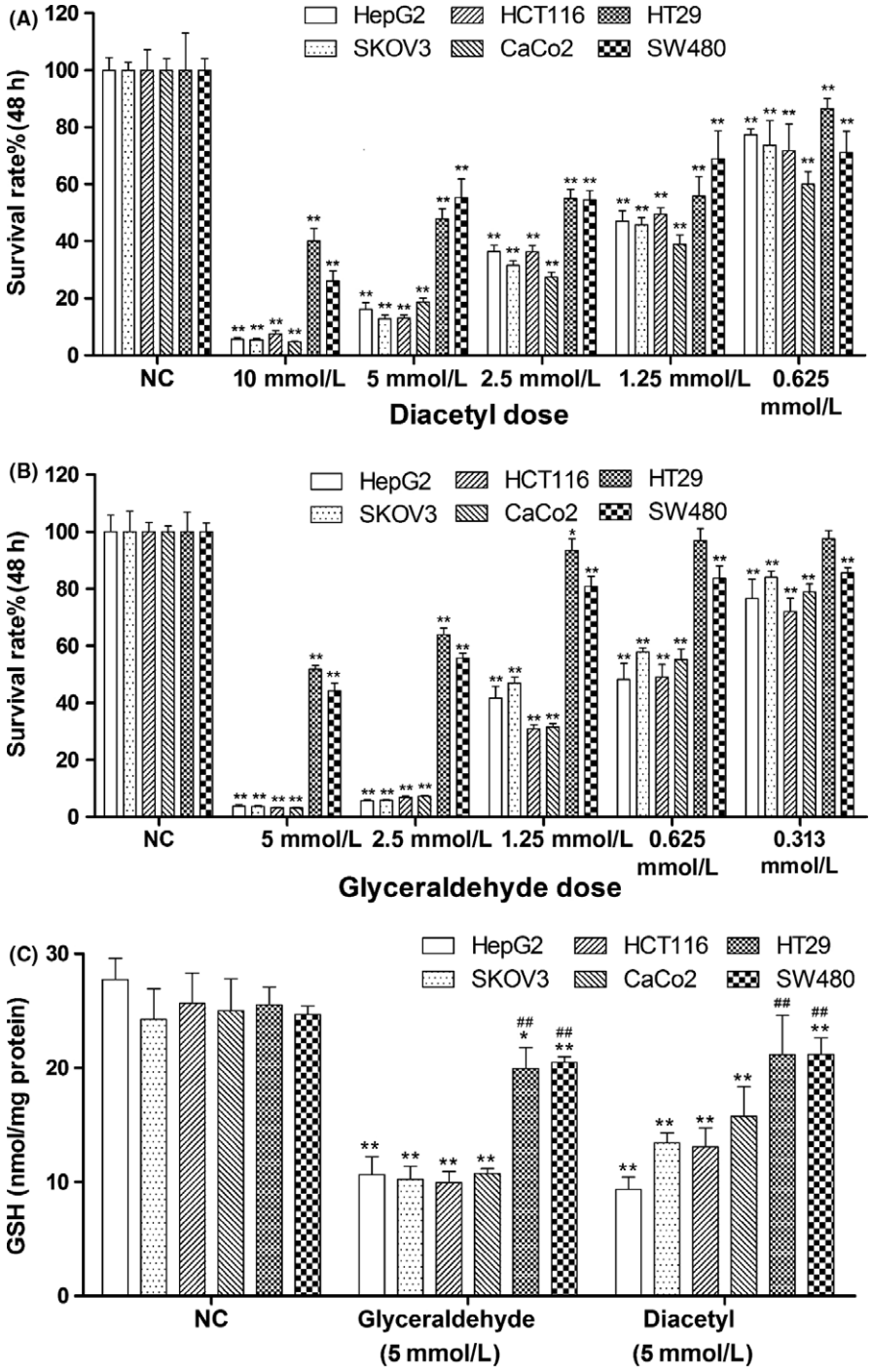

FIGURE 4 Cytotoxicity of glyceraldehyde and diacetyl on cancer cells. A, Cells treated with diacetyl. B, Cells treated with glyceraldehyde. C, Glutathione (GSH) levels were measured in cells treated with glyceraldehyde (5 mmol/L) and diacetyl (5 mmol/L). Bars represent mean ± SD (n = 5). $^{*}P < .05$; $^{**}P < .01$ vs NC group; $^{\#\#}P < .01$ vs HepG2, SKOV3, HCT116 or CaCo2 group. NC, normal control

5. Other AKR1Bs substrates also preferentially killed cells that overexpress these 2 enzymes.
6. Inhibiting the synthesis of GSH enhanced the toxicity of glyceraldehyde and diacetyl and, conversely, increasing the synthesis of GSH protected the cells against these drugs. These results indicate that, similar to 2DG, the substrates of other AKR1Bs also kill cells by depleting GSH.
7. Potency of the cytotoxicity of the substrates of AKR1Bs is directly correlated to how efficiently they are being catalyzed by the AKR1Bs.

A previous report pointed out that even though 2FDG is a more potent glycolysis inhibitor than 2DG, it is less effective in killing cancer cells than 2DG under normoxic conditions. The authors of this report suggested that 2DG does not kill cancer cells by inhibiting glycolysis, but by interfering with *N*-linked glycosylation.[7] Herein, we provide an alternative mechanism to explain the differential toxicity of 2DG and 2FDG to cancer cells. Kinetic analysis showed that 2DG is a much better substrate for AKR1B1 than 2FDG.[26] Thus, in cancer cells that overexpress AKR1B1, 2DG is more effective in depleting GSH than 2FDG, accounting for its more potent anticancer effect

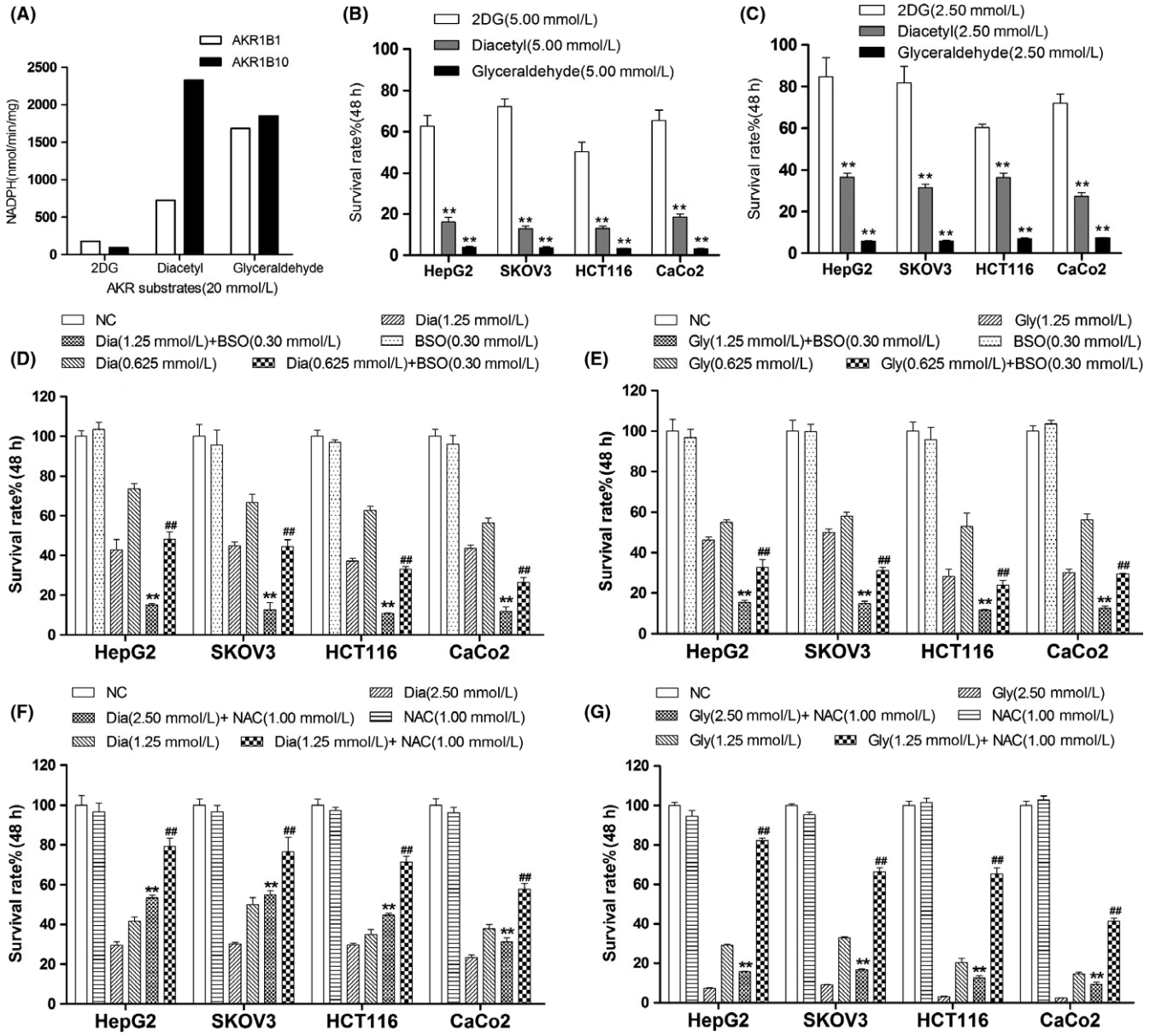

FIGURE 5 Cytotoxicity of AKR substrates depends on their catalytic efficiency of AKR1B1/AKR1B10 and the cellular levels of glutathione (GSH). A, Enzyme activity assay. B,C, Comparison of the toxicity of 2-deoxyglucose (2DG), diacetyl and glyceraldehyde, redrawn with part of the data from Figure 4 and Figure S1. $^{**}P < .01$ vs 2DG group. D, Effects of DL-buthionine-sulfoximine (BSO; Sigma Chemical Co.) on diacetyl (Dia) toxicity, $^{**}P < .01$ vs Dia (1.25 mmol/L) group; $^{\#\#}P < .01$ vs Dia (0.625 mmol/L) group. E, Effects of BSO on glyceraldehyde (Gly) toxicity, $^{**}P < .01$ vs Gly (1.25 mmol/L) group; $^{\#\#}P < .01$ vs Gly (0.625 mmol/L) group. F, Effects of *N*-acetyl-cysteine (NAC; Sigma) on diacetyl toxicity, $^{**}P < .01$ vs Dia (2.5 mmol/L) group; $^{\#\#}P < .01$ vs Dia (1.25 mmol/L) group. G, Effects of NAC on glyceraldehyde toxicity, $^{**}P < .01$ vs Gly (2.5 mmol/L) group; $^{\#\#}P < .01$ vs Gly (1.25 mmol/L) group. Bars represent mean ± SD (n = 5)

than 2FDG. It is likely that under hypoxic conditions, 2DG might kill cancer cells by inhibiting glycolysis, but under normoxic conditions, it kills cancer cells by AKR1Bs-mediated depletion of GSH. We cannot rule out the possibility that perturbation of *N*-linked glycosylation may also contribute to its anticancer effect under normoxic conditions.

Two of the AKR1Bs substrates, glyceraldehyde and diacetyl, were tested in a nude mouse xenograft tumor model. For convenience, the drugs were given by daily single dose injection by the tail vein. Although the availability of these drugs in vivo had not been optimized, results showed that they can retard tumor growth. These 2 drugs, particularly glyceraldehyde, are relatively non-toxic. Sustained continuous delivery of these drugs probably would have shown their anticancer effects more dramatically.

We showed that cytotoxicity of 2DG and the substrates of other AKR1Bs was a result of depletion of GSH. GSH is the major cellular antioxidant and its role in cell defense against endogenously generated or exogenously introduced reactive oxygen species (ROS) is well established. Cellular balance of GSH/GSSH ratio is important not only for defense against oxidative stress, but also for cell

TABLE 1 Acute toxicity experiment of diacetyl or glyceraldehyde in mice by a single iv injection (n = 8, half male and female)

Drugs	Dose (mg/kg BW)	Death/total	Mortality rate %	LD_{50} (mg/kg BW)
Diacetyl	300	0/8	0	410
	360	3/8	37.5	
	432	5/8	62.5	
	518	7/8	87.5	
	622	8/8	100.0	
Glyceraldehyde	1000	0/8	0	>2000
	2000	0/8	0	

BW, body weight; LD_{50}, median lethal dose.

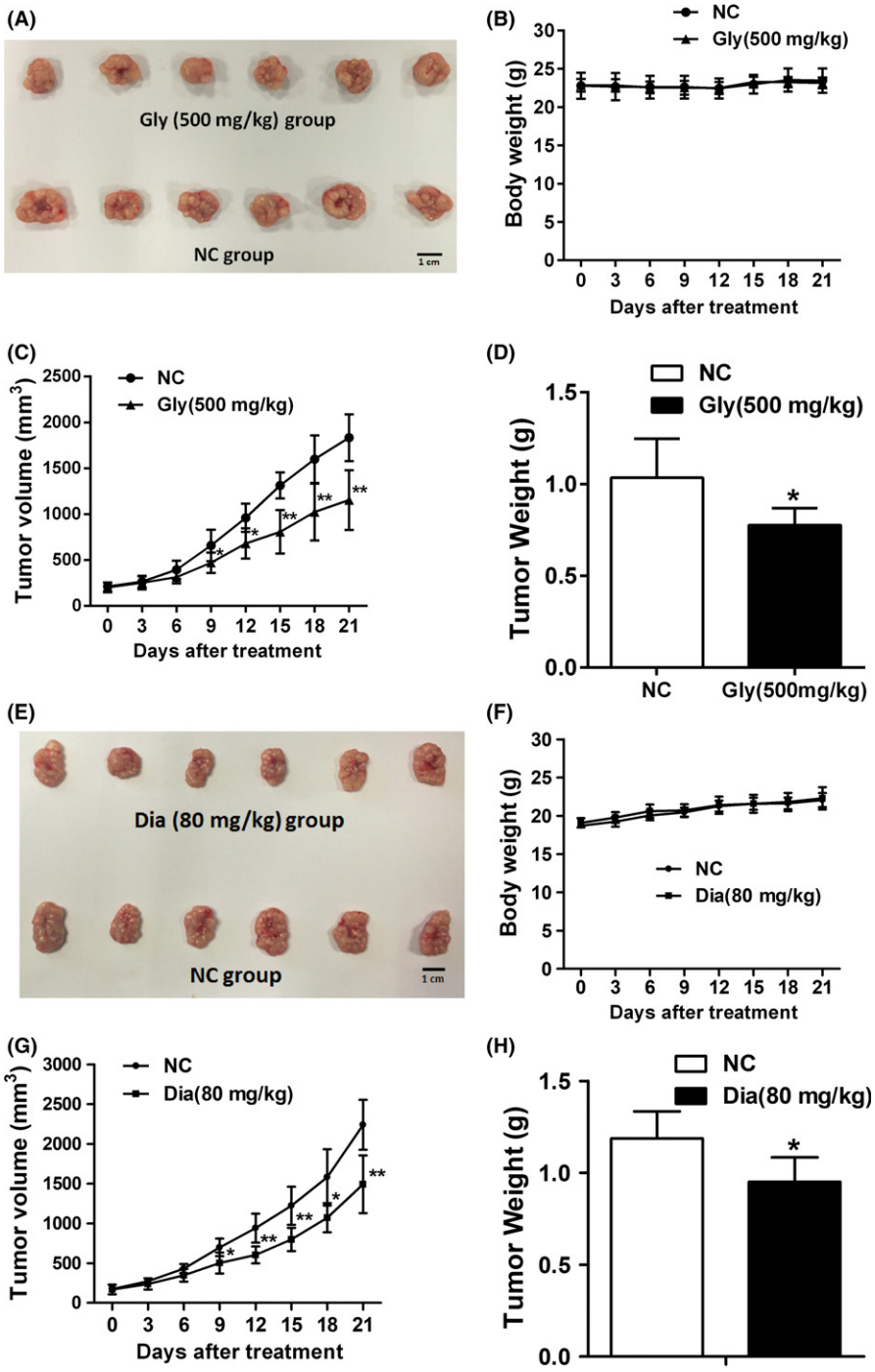

FIGURE 6 Anticancer effects of glyceraldehyde and diacetyl on tumor xenograft model. A-D, Anticancer effect of glyceraldehyde. A, Tumors. B, Body weight curve. C, Tumor volume. D, Tumor weight. E-H, Anticancer effect of diacetyl. E, Tumors. F, Body weight curve. G, Tumor volume. H, Tumor weight. Six nude mice were measured for statistical analysis in each group under the same conditions. *$P < .05$, **$P < .01$ vs NC group. Bars represent mean ± SD (n = 6). Gly, glyceraldehyde; Dia, diacetyl; NC, normal control

survival.[27,28] High levels of GSH are associated with resistance to apoptosis, whereas low levels induce apoptosis. It is likely that severe depletion of GSH caused by excessive activities of AKR1Bs would lead to cell death. GSH also plays a very important role in the development of cancers and in the treatment of cancers. High levels of GSH are required for tumor cell proliferation[29] and metastasis[30] High levels of GSH also confer resistance of cancer cells to chemotherapy[31] and radiotherapy.[32] Various strategies to deplete GSH in cancer cells to sensitize them to chemotherapy and radiotherapy have been suggested, including blocking the regeneration of GSH from GSSH, inhibiting the synthesis of GSH, and increasing the efflux of GSH from cells.[33] However, these treatments would also deplete GSH in normal cells, leading to undesirable side-effects. Targeted depletion of GSH in cancer cells can be achieved by exploiting the overexpression of AKR1Bs in these cells. In the presence of suitable substrates, their activities would deprive GR of NADPH for its regeneration of GSH. Augmentation of chemotherapy and radiotherapy of 2DG is probably based on this mechanism. The results of our studies reported herein predict that better substrates of ARK1Bs such as glyceraldehyde and diacetyl should be much more effective than 2DG in the augmentation of chemotherapy and radiotherapy.

Drugs based on our proposed mechanism can be used to treat several types of cancer. AKR1Bs are often overexpressed in a variety of cancers, including liver, prostate, breast, ovarian, cervical, rectal, lung and oral cancers.[34-39] Expression levels of these 2 enzymes in cancer cells vary, ranging from a <2-fold increase to an over 50-fold increase.[13] Those with high levels of 1 or both of these enzymes would be more susceptible to the toxicity of their substrates. Importantly, it has been reported earlier,[23] and shown here that the level of expression of AKR1Bs can be increased by bortezomib or MG-132. Therefore, even cancer cells with a low level of expression of AKR1Bs can be made susceptible to the anticancer effects of their substrates by increasing their levels of expression. This would greatly expand the types of cancer treatable by AKR1Bs substrates. Bortezomib and MG-132 are inhibitors of the ubiquitin-proteosome pathway and have been shown to increase the expression of Nrf2-regulated genes,[40] among them AKR1B1 and AKR1B10.[41] Bortezomib has been used to treat some cancers and to enhance other chemotherapies because improper degradation of regulatory proteins is thought to contribute to cancer growth.[42] It will be interesting to find out whether increased levels of AKR1Bs induced by this drug might also contribute to its anticancer effect because endogenous metabolites such as methyl glyoxal, and 4-hydroynonenal are excellent substrates for these 2 enzymes.

In this report, we show that we have identified a novel anticancer mechanism based on the activities of AKR1Bs. This would lead to the development of a new class of anticancer drugs. AKR1B1 and AKR1B10 can reduce a broad range of substrates, primarily small molecular weight aldehydes and ketones. We have indicated here that 2 of their substrates, glyceraldehyde and diacetyl, are good candidates for anticancer drugs. They should be more effective than 2DG in killing cancer cells. We believe that even better anticancer drugs can be developed. The high-resolution crystal structures of these 2 enzymes are known and their catalytic sites have been identified. It should be possible to design better substrates for these enzymes to serve as anticancer drugs. Substrates preferred by AKR1B10 might be better anticancer drug candidates because, unlike AKR1B1, which is expressed in all tissues, AKR1B10 is normally only expressed in the small intestine and colon.[12] This should restrict the potential undesirable side-effects of the drugs. Interestingly, another group of enzymes in the family of aldo-keto reductases, AKR1C1, -C2, and -C3, are also found to be overexpressed in breast, cervical, and prostate cancers.[43-45] These enzymes also use NADPH as their cofactor. Therefore, their substrates could also be used to treat cancers that overexpress these enzymes.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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