

Diazoxide-Mediated Growth Inhibition in Human Lung Cancer Cells via Downregulation of β -Catenin-Mediated Cyclin D1 Transcription

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Abstract Treatment of various types of cells with the mitochondrial ATP-sensitive K^+ channel opener, diazoxide, preconditions cells to subsequent injuries and inhibits apoptosis. However, the role and mechanism(s) of diazoxide in solid tumor cell growth are largely unknown. Here we demonstrate that diazoxide inhibited the proliferation of lung cancer cells as well as the transcription of cell cycle-related protein Cyclin D1. Cyclin D1 overexpression inhibited the negative role of diazoxide in cell cycle progression. We further explored the mechanisms by which diazoxide affected Cyclin D1 transcription and found that the β -catenin transcription factor was downregulated by diazoxide, with a corresponding decrease in Cyclin D1 promoter activity. Taken together, these results suggest that diazoxide inhibits lung cancer cell proliferation via downregulation of Cyclin D1 transcription, which may have important therapeutic implications in lung cancer patients.

Keywords β -Catenin · Diazoxide · Lung cancer · Cyclin D1 · Transcriptional regulation

Introduction

In various types of cells, opening of the mitochondrial ATP-sensitive K^+ (mitoK-ATP) channels leads to enhanced resistance to subsequent injury caused by dif-

ferent stimuli, including induction of apoptosis [1–4]. MitoK-ATP channels can be selectively opened using low doses of diazoxide [5]. Diazoxide is a potassium channel activator, which causes local relaxation in smooth muscle by increasing membrane permeability to potassium ions [4–9]. Although the effect of diazoxide on isolated mitochondria has been thoroughly studied, the role and mechanism(s) of diazoxide in solid tumor cell growth are largely unknown. Therefore, the goal of our study was to explore the morphologic and biochemical changes induced by diazoxide in tumor cells and find out how such changes might lead to enhanced prosurvival signaling.

One of the main properties of cancer cells is their increased and deregulated proliferative activity [10, 11]. It is now well known that abnormalities in many positive and negative modulators of the cell cycle are frequent in many cancer types, including lung carcinomas [12, 13]. Cyclin D1, a member of the Cyclin D family, regulates the initial G1-to-S transition by inhibiting Rb and activating E2F proteins, and it contributes to cell apoptosis, proliferation, and differentiation [14]. Cyclin D1 is amplified and/or overexpressed in a number of human cancers, including breast carcinoma and B cell and T cell malignancies, and contributes to tumor cell growth [14–16]. Accumulated evidence indicates that there is extensive cross-talk between the transcriptional and posttranscriptional regulation of Cyclin D1 expression and its biological functions.

In this study we examined the effect of diazoxide on H1299 cell proliferation. Diazoxide inhibited the proliferation of H1299 cells as well as the transcription of cell cycle-related protein Cyclin D1 via downregulation of β -catenin transcription factor, which provided a new mechanism of diazoxide-involved cell proliferation and may have important therapeutic implications in lung cancer patients.

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Methods

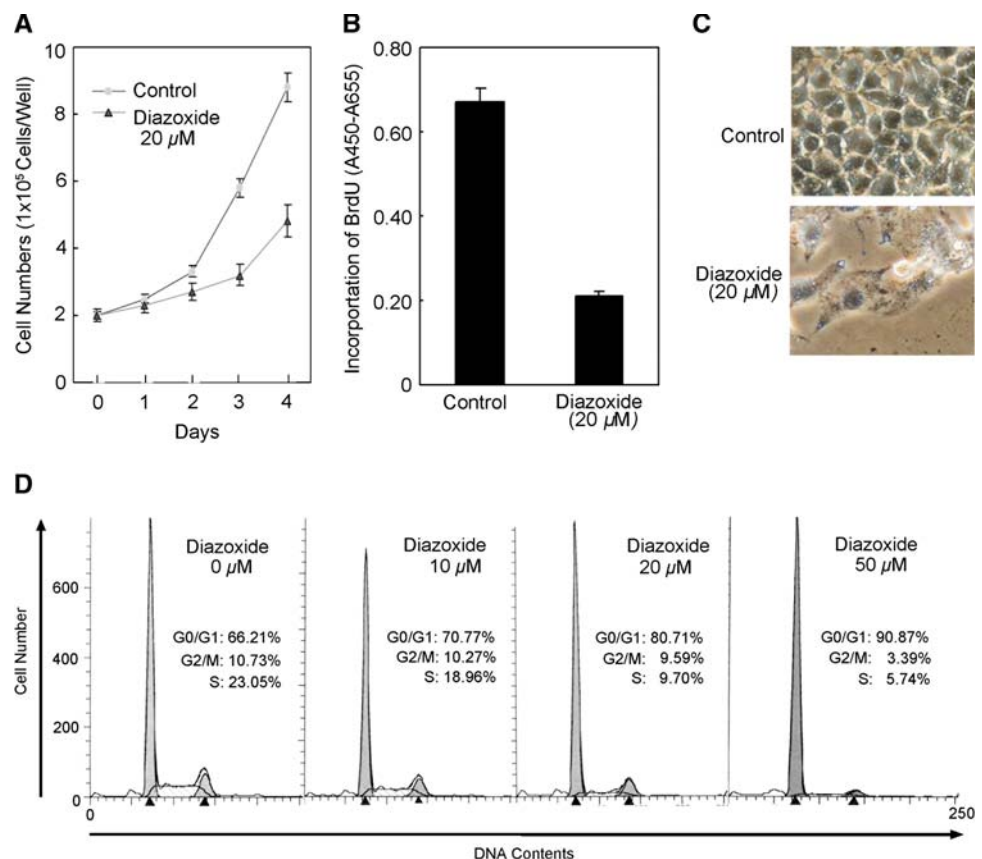
Materials

Restriction enzymes, bovine calf serum, RPMI-1640 medium, Trizol reagent, and the mammalian expression vector pcDNA3.0 were from Invitrogen (Carlsbad, CA, USA). Diazoxide and aprotinin were from Sigma Chemical Co (St. Louis, MO, USA). Anti-human-Cyclin D1, anti-human-Cyclin D2, anti-human-Cyclin D3, anti-human-CDK4, anti-human-CDK6, and anti-human- β -catenin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-myc and anti-actin antibodies were purchased from Oncogene (Cambridge, MA, USA). Anti-mouse-HRP secondary antibody and anti-rabbit-HRP secondary antibody were purchased from New England BioLabs (Ipswich, MA, USA). Other reagents were commercially available in China. The enhanced chemiluminescence (ECL) assay was from Amersham Pharmacia Biotech (Uppsala, Sweden). TaKaRa RNA PCR Kit (AMV ver.2.1) and TaKaRa MutanBEST kit were from TaKaRa Bio Inc. (Otsu, Shiga, Japan).

Plasmids

Expression constructs for EGFPN3, pcDNA3.1-myc-A, pGL3Basic, and pRL-CMV were kindly provided by

Fig. 1 Effect of diazoxide on the proliferation of H1299 cells. **a** H1299 cells treated with control or diazoxide (20 μ M) for 24 h were trypsinized and plated at densities of 2×10^5 into 6-well plates (Day 0). Cell numbers were counted every 24 h. Results represent the mean of at least four independent experiments; bar = SE. **b** BrdU uptake assays as an index of DNA synthesis were performed with H1299 cells treated or not treated with diazoxide (20 μ M) for 24 h, as described in Methods. Results are expressed as the mean \pm SD of five independent experiments. **c** Compared to control, H1299 cells treated with diazoxide (20 μ M) for 24 h had a markedly reduced number of cells. **d** Exponentially growing cells were treated with the indicated concentrations of diazoxide. H1299 cells were stained with propidium iodide 48 h after diazoxide exposure



Dr. Dan Liu (Shanghai Medical College of Fudan University). Full-length cDNA of Cyclin D1 was generated using PCR with 0.1 μ g human fetal liver cDNA library as the template, and the primers used were primer 1 (sense) (5'-TATGGAATTCCCCAGCCATGGAA-3') and primer 2 (antisense) (5'-TATGGATCCCGCCCTCAGAT-3'). The PCR product was inserted in-frame into the pcDNA3.1-mycA vector via *Eco*RI and *Bam*HI restriction sites. Clones were sequenced to identify the right insert. A 1800-bp fragment of the human Cyclin D1 promoter was isolated by PCR using 1 μ g human genomic DNA as the template as previously described [17]. Reporter genes containing sequentially truncated fragments (-1745/+18, -963/+18, -680/+18, -420/+18, -240/+18, -160/+18, -140/+18, and -60/+18) of the Cyclin D1 promoter region were prepared in a similar manner. Cyclin D1 promoter LEF1 binding-site-directed mutagenesis constructs were derived from pGL3(-420/+18) by PCR amplification using TaKaRa MutanBEST mutagenesis kit.

Cell Culture and Transfection

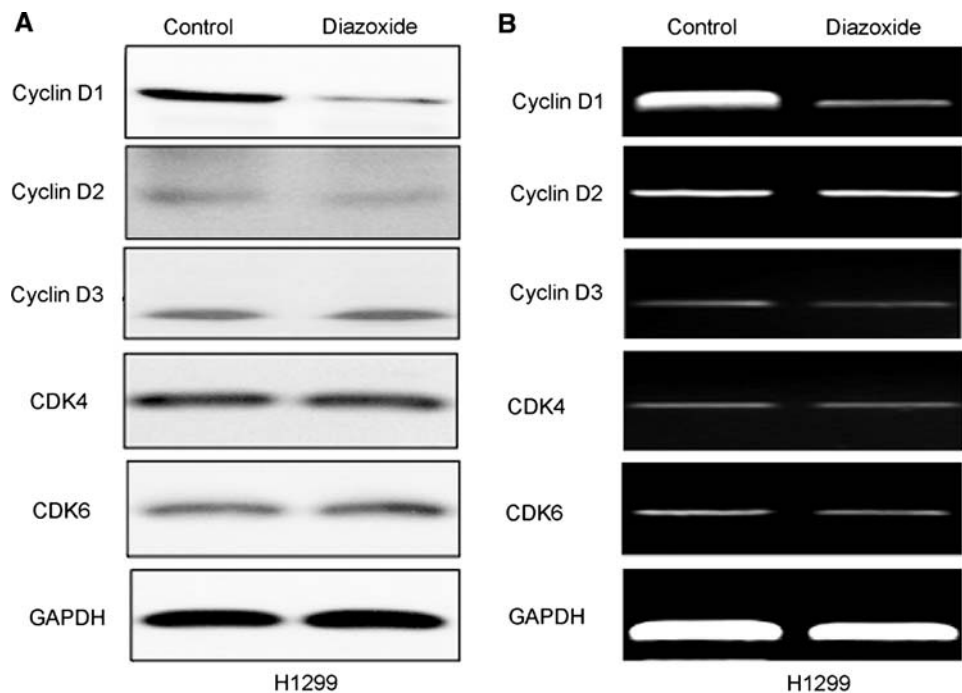
H1299 cells were purchased from Shanghai Institutes for Biological Sciences and cultured in Dulbecco's modified Eagle's medium. Cell transfection was performed with

Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Analysis of Cell Cycle by FACS and Western Blot Analysis

After 48 h of transfection, adherent and nonadherent cells were collected, washed twice in phosphate-buffered saline (PBS), and fixed with ice-cold 70% ethanol for at least 1 h. The fixed cells were washed and stained with propidium iodide mixture containing 50 $\mu\text{g/ml}$ propidium iodide, 0.05% Triton X-100, 37 $\mu\text{g/ml}$ EDTA, and 100 U/ml ribonuclease in PBS. After incubation for 45 min at 37°C, the DNA content was determined by quantitative flow cytometry with standard optics of the FACScan flow cytometer (FACStar, Becton, Dickinson, Franklin Lakes, NJ, USA) as previously reported [18]. Western blot was performed as previously described [19] using an antibody to GAPDH to ensure equivalent loading.

Fig. 2 Effect of diazoxide on the expression of protein-regulating cell cycle. **a** Cells treated with diazoxide (20 μM) for 24 h were examined for the expression of molecules regulating apoptosis by Western blot analysis using specific antibodies. GAPDH was used as an internal control. **b** Cells treated with diazoxide (20 μM) for 24 h were examined for the expression of molecules regulating apoptosis by RT-PCR using specific primers. GAPDH mRNA expression was used as an internal control. **c** H1299 cells were transiently transfected with Cyclin D1 promoter construct pGL3(-1745/+18). At 24 h after transfection, cells were treated with vehicle or an increasing dose of diazoxide for an additional 24 h. The luciferase activity values were standardized to those observed in nontreated samples. Each value is the mean \pm SD of at least three independent experiments



Dual Luciferase Assay and Reverse Transcription (RT)-PCR

Dual luciferase assay and reverse transcription (RT)-PCR were performed as described [19]. Primers used for PCR were as follows: Cyclin D1-F, 5'-CCGTCCATGCGGAA GATC-3' and Cyclin D1-R, 5'-ATGGCCAGCGGGAA GAC-3'.

Results

Effect of Diazoxide on Cell Proliferation in H1299 Cells

We first examined the effect of diazoxide on the cell proliferation of H1299 cells. Diazoxide dramatically reduced the proliferation rate in a dose-dependent manner compared to untreated cells (Fig. 1a). Consistent with the inhibition of

cell proliferation, DNA synthesis, as determined by BrdU incorporation, was also significantly reduced in H1299 cells treated with diazoxide compared with that of control (Fig. 1b). Compared to control, H1299 cells treated with diazoxide (20 μ M) for 24 h had a markedly reduced number of cells (Fig. 1c). The effect of diazoxide on the cell cycle was determined in H1299 cells using FACS analysis. As shown in Fig. 1d, diazoxide induced a G1 phase arrest in a dose-dependent manner (Fig. 1d).

Diazoxide Downregulated Cyclin D1 mRNA Expression

To address the mechanisms of diazoxide-reduced cell growth, we investigated the effect of diazoxide on the expression of the Cyclin D family proteins (Cyclin D1, D2, and D3) and their corresponding kinases (CDK4 and CDK6), which are involved in the regulation of cell cycle progression and mitogenic signals [14, 20]. As depicted in Fig. 2a, diazoxide downregulated the expression of Cyclin D1 without significantly changing the expression of the other examined proteins. Next, we performed RT-PCR to

determine the effects of diazoxide on the mRNA expression of the Cyclin D family, CDK4, and CDK6. As depicted in Fig. 2b, diazoxide inhibited the mRNA expression of Cyclin D1. There were no significant differences in Cyclin D2, Cyclin D3, CDK4, or CDK6 mRNA levels before and after diazoxide treatment. To determine the role of diazoxide in Cyclin D1 transcription, we constructed Cyclin D1 promoter pGL3 that contained the region from nucleotide position -1745 to $+18$ relative to the transcription start codon. Transfection studies using pGL3 treated with the indicated dose of diazoxide showed that diazoxide inhibited the activity of the Cyclin D1 promoter in a dose-dependent manner in H1299 cells (Fig. 2c).

Diazoxide Inhibited Cyclin D1 Transcription via Downregulation of β -Catenin Transcription Factor

To identify the *cis* element responsible for the effect of diazoxide, luciferase reporter constructs containing progressive deletion of the 1745-bp genomic DNA fragment were generated and cotransfected into H1299 cells treated or not treated with diazoxide. The luciferase assay showed

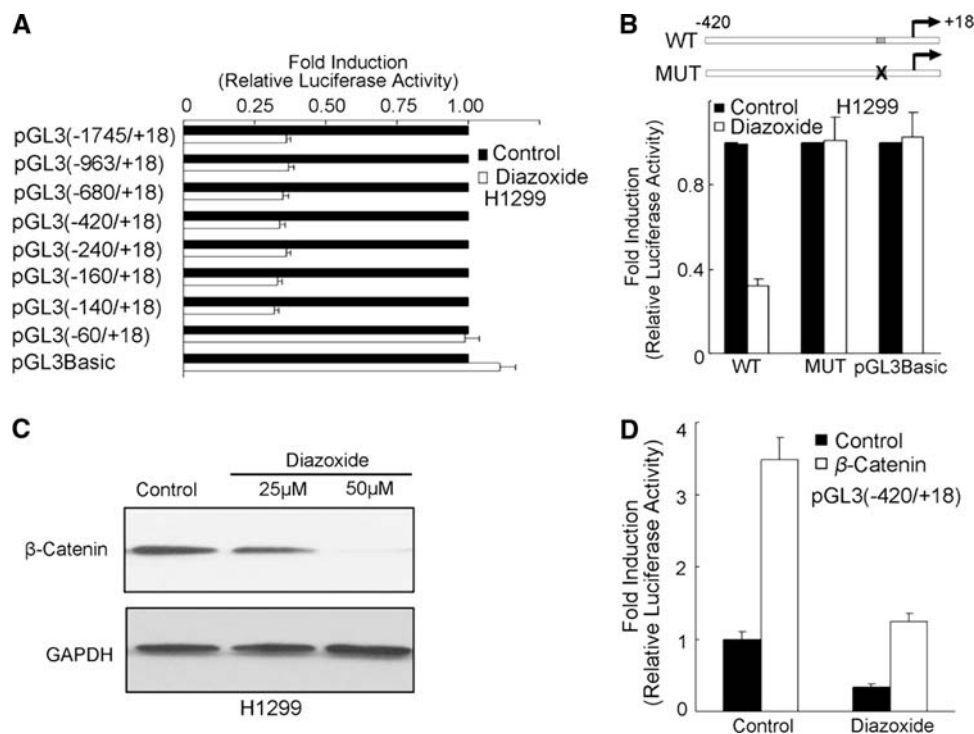


Fig. 3 Diazoxide downregulates Cyclin D1 promoter activity by reducing the level of transcription factor β -catenin. **a** Cyclin D1-luc constructs containing various lengths of Cyclin D1 promoter regions were transiently transfected into H1299 cells treated or not treated with diazoxide. Luciferase activities were measured as described above. Normalized luciferase activity was standardized to that with vector alone. **b** Reporter plasmid pGL3($-420/+21$) (WT) or its LEF-1 binding-site-mutated constructs (MUT) were transfected into H1299 cells. At 24 h after transfection, cells were treated with control or

diazoxide for 24 h. The luciferase activities were obtained and presented as described above. **c** H1299 cells were treated for 24 h with indicated dose of diazoxide and cell extracts were subjected to immunoblot analysis using an anti- β -catenin antibody. **d** H1299 cells were transiently cotransfected with pGL3($-420/+21$) construct and control vector or β -catenin expressing vector. At 24 h after transfection, cells were treated or not treated with diazoxide for 24 h. Normalized luciferase activity was standardized to pGL3($-420/+18$) with control vector in untreated cells

that a deletion from –140 to –60 bp resulted in a loss of diazoxide activation (Fig. 3a).

Cyclin D1 is a key molecule in mitogenic signaling and mediates S-phase entry from the G1 phase in the cell cycle [15]. The Cyclin D1 promoter contains a typical T-cell-factor (Tcf)/lymphoid-enhancer-factor (LEF)-1 binding site and its activity is largely dependent on β -catenin [21]. Inspection of this region between –140 and –60 revealed one identified LEF-1-binding site [22]. To investigate whether the inhibitive effect of diazoxide upon the Cyclin D1 promoter was mediated by the LEF-1-binding site, we introduced site-directed mutagenesis into this LEF-1-binding site on the pGL3(–420/+18) reporter plasmid as previously described. It was found that the mutagenesis of this LEF-1-binding site abolished the effects of diazoxide on the Cyclin D1 promoter activity (Fig. 3b). In addition, a lower level of β -catenin was observed in extracts prepared from diazoxide-treated cells (Fig. 3c), suggesting that diazoxide may downregulate Cyclin D1 transcription via decreasing the β -catenin level. This conclusion was further supported in Fig. 3d, as the stimulation of Cyclin D1 promoter activity by transient transfection with β -catenin expression plasmids was largely blunted after diazoxide treatment.

Cyclin D1 Overexpression Abrogated Diazoxide-Induced G1 Arrest

Because diazoxide downregulated Cyclin D1 expression, we wanted to determine whether Cyclin D1 played an important role in diazoxide-induced G1 arrest. To address this point, H1299 cells were transfected with control or GFP-tagged Cyclin D1 plasmid (Fig. 4a). Cyclin D1 overexpression abrogated diazoxide-induced G1 arrest by flow cytometry (Fig. 4b).

Discussion

In recent studies, the understanding of how diazoxide inhibited cell growth in hematologic cancers has greatly improved [5, 7, 9]. However, the mechanisms of the diazoxide-mediated growth inhibition in solid cancer are not well understood. The aim of our investigation was to elucidate the role of diazoxide in cell proliferation. In this report, we have described several observations in which the role of diazoxide in H1299 cell growth has been implicated by FACS assay, cell number, and BrdU incorporation. The results of our study suggest that diazoxide may be clinically useful in lung cancer patients as an adjuvant chemotherapeutic agent.

To elucidate the molecular mechanism of the role of diazoxide in H1299 cell growth, we have demonstrated that

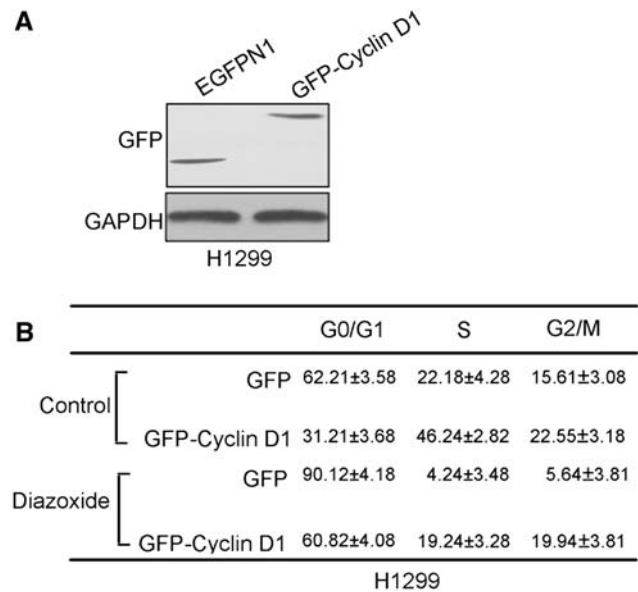


Fig. 4 Cyclin D1 overexpression abrogated diazoxide-induced G1 arrest. **a** H1299 cells were transiently transfected with control vector or GFP-tagged Cyclin D1 expression plasmid and cell extracts were analyzed by immunoblotting with an anti-GFP Ab. GAPDH served as a loading control. **b** Control or GFP-Cyclin D1 expression plasmid was transiently transfected into H1299 cells treated or not treated with diazoxide. At 24 h after transfection, the DNA content of cells was determined by propidium iodide staining and FACS analysis. The percentage of cells in each stage of the cell cycle is shown with each FACS profile

diazoxide decreases the protein and mRNA expression of Cyclin D1. Evidence of the oncogenic potential of Cyclin D1 is provided by studies with numerous models in which the elevated expression of Cyclin D1 shortens the G1 phase of the cell cycle and enhances malignant transformation [14, 23]. An overexpression of Cyclin D1 blocked diazoxide-decreased H1299 cell growth, demonstrating that Cyclin D1 plays a major role in diazoxide-inhibited cell proliferation in H1299 cells. The limitations of our study are listed as follows: (1) All the experiments were performed with H1299 cells. Thus, it is important to show that the results obtained represent a general phenomenon and not a peculiarity of H1299 cells. (2) All the experiments were performed with H1299 cells in vitro. To further investigate the effect of diazoxide on lung cancer cell growth, the effect of diazoxide on H1299 cell growth in vivo should be performed using tumorigenicity assay.

Another interesting finding is that diazoxide downregulated Cyclin D1 transcription via reduction of β -catenin transcription factor. β -Catenin is a well-known DNA-binding nuclear protein that is widely expressed in tissues and binds to GC box motifs in promoters of many house-keeping genes and genes involved in growth regulation and cancer, including p21, caspase-8, Cyclin D1, and GalT V [24–26]. Biologically, β -catenin plays important roles in a

wide variety of physiologic processes, including cell cycle, hormonal activation, apoptosis, angiogenesis, and oncogenesis [24, 27]. To our knowledge, this is the first report that diazoxide decreased β -catenin expression, which contributed in diazoxide-reduced Cyclin D1 transcription, which elicited a new mechanism of diazoxide-contributed tumor development.

In summary, this work suggests that diazoxide reduced cell progression in H1299 cells via downregulation of Cyclin D1 transcription in a diazoxide-dependent manner, indicating that diazoxide is a very promising new drug for use in H1299 cells. The molecular mechanism of the downregulation of β -catenin mediated by diazoxide should be next investigated.

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