

BRD9 controls Oxytocin signal pathway in gastric cancer via CACNA2D4 and CALML6

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Abstract

Background: First-line chemotherapeutic agents have great activation treatment in cancers, but the side effect of these kind drugs also hurt the healthy cells, in some cases, cancer cells are induced drug resistance to chemotherapeutic agents. The molecular mechanisms underlying such side effect have been studied in a range of cancer types, yet little is known in depressing the adverse effect of chemotherapeutic drugs by targeting BRD9 in gastric cancers.

Methods: We used two gastric cancer cell lines (MGC-803 and AGS) for comparison. We applied molecular and cellular techniques to measure cell survival, mRNA expression, investigated clinical data in the consensus of The Cancer Genome Atlas, and combined with the High-Throughput Sequencing approach in MGC-803 cells and AGS cells, for global gene expression analysis in inhibiting BRD9 conditions.

Results: Our studies have shown that cancer cells with BRD9 over-expression, MGC-803 cells, are more sensitive to BRD9 inhibitors, BI9564 or BI7273, than AGS cells. Its mechanism is related to the regulation of CACNA2D4 and CALML6 oncogenes in the Oxytocin signaling pathway. And BRD9 inhibitors can enhance the sensitivity of gastric cancer MGC-803 cells to adriamycin and cisplatin, so we may reduce the dosage of chemotherapeutic agents in curing gastric cancers with BRD9 over-expression by combining BI9564 or BI7273 with adriamycin or cisplatin.

Conclusions: Our study elucidates the feasibility and effectiveness of inhibiting BRD9 to reduce the adverse effect of first-line chemotherapeutic agents in treating over-expressed BRD9 gastric cancer, providing a scientific theoretical basis on chemotherapy regimen in over-expressed BRD9 gastric cancer.

Background

Producing adverse effects in curing cancer patients is a main problem in both novel targeted therapeutics and conventional chemotherapeutics [1]. Adriamycin (ADR, Doxorubicin, Dox) and cisplatin (CDDP), first-line chemotherapeutic medicines, are potent chemotherapeutic agents which are used for the treatment of numerous cancers[2, 3]. Adriamycin's mechanism of effect involves inhibiting the synthesis of DNA and damage to DNA[4], and the formation of reactive oxygen species (ROS) [5] to create oxidative stress in the cellular environment. The mechanism of cisplatin (CDDP) is related to DNA Double-stranded covalent crosslinking and forming DAN-cisplatin adducts [6]. Although the action mechanism of adriamycin and cisplatin are multiple, many healthy cells are hurt to some extent and some cancers are developing drug resistance to them too[6]. Damaged healthy cells in cancer patients will depress their immunity, bad for their recovering healthy. Many cancer patients congenital or acquired drug resistance to chemotherapy including ADR and CDDP. The ways of drug resistance of cancer cells include the increased expression of DNA repair genes, abnormal drug transport pathway, acetylation of histones, epigenetic modifications activating drug resistance pathway and so on[7, 8]. these phenomena seriously reduce the efficacy and anti-cancer spectrum of first-line chemotherapeutic drugs. So, the most urgent

and important problem in the clinical application of first-line chemotherapeutic drugs is downregulation these kinds of side effects.

Recently many studies showed abnormal epigenetic control is one of the important reasons in causing carcinoma's drug side effects. In many carcinoma's types, epigenetic regulation involving gene expression, DNA repair, and DNA replication [9, 10]. Epigenetic regulation protagonists include writers, readers, and erasers, as well as members of chromatin-remodeling complexes, mutations in these genes, are pervasive in cancer, and few if any cancers escape mutations in one of these major chromatin rheostat proteins. Some studies showed that members of the mammalian SWI/SNF chromatin-remodeling complex are mutated in more than 20% of all cancers [11]. These mutations and alongside mutations in histones themselves can promote the development of malignancy and resistance to drugs in cancer cells [12]. These findings firmly establish that epigenetic dysregulation plays a causal role in cancer initiation, progression and developing drug resistance. On the other hand, the drugs targeting epigenetic regulator have fewer side effects than chemotherapeutic drugs' in healthy cells. Because most chemotherapeutic agents targeting oncogene DNA, many healthy cells' DNA may be hurt by chemotherapeutic agents either.

As epigenetic readers, bromodomain proteins can recognize acetylated histone tails to facilitate the transcription of target genes. Based on structural conservation the human bromodomains, approximately 60 as we knew, can be divided into eight sub-families. The family IV of bromodomain-containing proteins include seven members (BRPF1, BRPF2, BRPF3, BRD7, BRD9, ATAD2, and ATAD2b) [13]. While the bromodomains of BRD7 and BRD9 are members of the SWItch/Sucrose Non-Fermenting (SWI/SNF) chromatin remodeling complex, which regulates gene expression, as showed in figure 1A. BRD9 has been shown to recognize the doubly acetylated histone H4K5acK8a, the di-propionylated ligand H4K5prK8pr (Figure 1B, Figure 1C) and histone H4K5buK8bu [14].

We selected gene BRD9 through consensus of The Cancer Genome Atlas (TCGA). In clinical cases and cancer cells, mutations of BRD9 are common, Cell lines data from TCGA database shows 21% of cancer cells mutate in BRD9 (Figure 1D). Some study shows that BRD9 abnormal expression is related to cervical cancer, non-small cell lung cancer and liver cancer [15–17]. This kind of mutation is also found in endometrial cancer, squamous cell lung cancer and prostate adenocarcinoma [18]. One study showed that PC9 cell mutated in BRD9 was drug resistance to EGFR inhibitor [19]. We can see that gene BRD9 mutation is common, and up to now none study investigated the role of BRD9 in gastric cancer.

Data from the TCGA database shows 26% mutation was found in gastric cancer (Figure 2A). Terry D. Crawford's study showed that BRD9 inhibitor decreased BRD9 binding to chromatin, and prevented the emergence of a drug-resistant population in EGFR mutant PC9 cells treated with EGFR inhibitors [19]. Anja F. Hohmann and his team found that the BRD9 and the SWI/SNF chromatin remodeling complex are hyperactive in acute myeloid leukemia (AML) cells, they sustain MYC transcription, rapid cell proliferation, and a block in differentiation. Inhibiting BRD9 can reverse the proliferation of cancer cells induced by

SWI/SNF [20]. Notwithstanding SWI/SNF composition and BRD9 were studied in these two kind cancer cells, the role of BRD9 remains to be studied in gastric cancer.

The incidence of gastric cancer is highest in Eastern Asia[21], to find the effective way for BRD9 over-expressed gastric cancer treatment, it is necessary to study the role of BRD9 in this kind of cancer. Here we show that BRD9 promotes CACNA2D4 and CALML6 expression in the Oxytocin signal pathway and induces gastric cancer cells proliferation. AGS cells and MGC-803 cells are two type of gastric cancer cells, the expression of BRD9 in MGC-803 cells is higher than AGS cells. MGC-803 cells are more sensitive to BRD9 inhibitors (BI9564 and BI7273) than AGS cells. The results described here reveals the potential signaling pathway controlled by BRD9 in BRD9 over-expressed gastric cancer contexts. And we found that when we combining BI9564 or BI7273 with adriamycin or cisplatin to treat these two types gastric cancer cells, the dosage of adriamycin or cisplatin needed by MGC-803 cells was downregulated greatly. The data and analyses provide the feasibility and effectiveness of inhibiting BRD9 to reduce the adverse effect of first-line chemotherapeutic agents in treating BRD9 over-expressed gastric cancer, providing a scientific theoretical basis on chemotherapy regimen in gastric cancer.



Figure 1 Information of BRD9

A: The BRD7/9 bromodomain-containing proteins are subunits in the SWI/SNF complexes; B: Coordination of H4K5prK8pr by the BRD9 bromodomain; C: Surface representation of the BRD9 bromodomain dimer in complex with H4K5prK8pr (Monomers colored in green and blue); D: Landscape of genomic aberrations in BRD9 gene in cancer cells.

Methods

Chemicals and reagents

We bought 3(4, 5dimethylthiazol2yl)2,5diphenyltetrazolium bromide (MTT) from Sigma-Aldrich (St Louis, MO), PrimeScript RT reagent Kit and SYBR Premix Ex Taq TM from TaKaRa.E. Z. N.AR HP Total RNA Kit, the product of Omega Bio-Tek (Doraville, USA) [22]. Adriamycin (ADR) and cisplatin were bought from Zhejiang HISUN Pharmaceuticals Co. (Zhejiang, China) [22].

Cell culture

AGS cell lines and MGC-803 cell lines were cultured by our lab. 10% fetal bovine serum and RPMI 1640 medium (Gibco BRL) were added in these two kind cells, and the cells were cultivated under a humidified 5% CO₂ atmosphere 37°C.

MTT assay

We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; SigmaAldrich, St. Louis, MO, USA) assay to determine drug sensitivity. AGS and MGC-803 cells were seeded into 96 well plates at a concentration of 5×10^3 cells/200 μ l/well. Cells were hatched at 37°C in a humidified 5% CO₂ incubator. Following 48 h treatment with specific concentrations of the anticancer drugs ADR or CDDP, BI9564 or BI7273 (Purchased from Selleck.) plates were added to standard tissue incubator conditions. Medium was removed and cells were solubilized in 150 μ l DMSO. The intensity of formazan was measured at 490 nm using an automated microplate spectrophotometer (iMark; BioRad, Hercules, CA, USA). The viability was calculated as (OD value of the treated group/OD value of untreated group) x 100%. We performed three times in every experiment.

Analysis of drug sensitivity activity

The viability of AGS and MGC-803 cells following treatment with ADR or CDDP in the presence or absence of BI9564 or BI7273 (500nm or 1000nm) were analyzed by MTT assay. Following plotting of the dose-response curve, the IC₅₀, the concentration of drug inhibiting 50% of cells was calculated.

Quantitative real-time PCR

After dealing as indicated, total mRNA of cells was extracted with TRIZOL reagent. The first strand of cDNA synthesis was generated from 2 μ g total RNA using oligo-dT primer and Superscript II Reverse Transcriptase (GIBCO BRL, Grand Island, NY, USA). Quantitative Real-Time PCR was carried on an iCycler (Bio-rad, Hercules, USA) using confirmed primers and SYBR Premix Ex Taq II (Takara, Japan) for detection. The cycle number when the fluorescence first reached a preset threshold (Ct) was used to quantify the initial concentration of individual templates for expression of mRNA of genes of interest. Primer pairs were as follows: BRD9, forward 5'- GCGACTTGAAGTCGGACGAGAT-3' and reverse 5'- GTCCACCACTTTCTTGCTGTAGC-3'; CACNA2D4, forward 5'- CCAACAATGGCTACATCCTCTCC-3' and reverse 5'- GATTCAGCCTGGTCTTCCCACT -3'; CALML6, forward 5'- GGGCTACATTGACTGGAACACAC -3' and reverse 5'- CCTCATAGTCGATGGTCCTGTC -3'; GAPDH, forward 5'- GCA CCGTCAAGGCTGAGAAC -3' and reverse 5'-TGGTGA AGACGCCAGTGGA -3'.

Results

MGC-803 cells are BRD9 over-expressed cell model

As shown in figure 2A, there are 106 (26%) of 407 gastric cancer patients' BRD9 expression upregulated. The change of gene BRD9 was shown in figure 2B, there are 5% gastric cancer patients' gene BRD9 amplification. According to above analysis, we first choose six different types of gastric cancer cells, AGS, Fu97, MKN1, NCIN87, SNU1, to screen BRD9-overexpressed model. As shown in figure 2C, gene BRD9 expression amount in MGC-803 cells was higher than in AGS cells. To study the possibility of targeting

BRD9 to downregulate the adverse effect of chemotherapeutic drugs to patients with BRD9 over-expressed gastric cancer, we chose MGC-803 cells as a BRD9-overexpressed model and AGS cells as a control group.



Figure 2 Expression of BRD9 in gastric cancer

A: Landscape of genomic aberrations in BRD9 gene in 407 patients with gastric cancer; B: Changes of gene BRD9 in gastric cancer patient; C: Results of RT-PCR in six kinds of gastric cancer cells

The cytotoxicity in MGC-803 cells of BRD9 inhibitors was stronger than in AGS cells.

Then we chose BRD9 special inhibitors, BI9564 and BI7273, to study what kind of effect will be when gene BRD9 was depressed. From figure 3A and figure 3B, we can see the molecular structures of BI9564 and BI7273 are similar.

Our study has shown that cancer cells with BRD9-overexpressed, MGC-803 cells, were more sensitive to BI9564 and BI7273 than AGS cells. By treating with BRD9 inhibitors, the therapeutic effect towards AGS cells and MGC-803 cells were investigated by MTT assay (Figure 3C, 3D, 3E, 3F). The IC₅₀ values of BI9564 in AGS cells and MGC-803 cells were 488.97 nm and 350.28 nm, respectively (Figure 3C, 3D). The BI9564's IC₅₀ in AGS cells is 1.4 times of that in MGC-803 cells. The IC₅₀ values of BI7273 on AGS cells and MGC-803 cells were 579.47 nm and 447.90 nm, respectively (Figure 3E, 3F). The BI9564's IC₅₀ in AGS cells is 1.3 times of that in MGC-803 cells. That is to say cancer cells with BRD9-overexpressed are more sensitive to BRD9 inhibitors, and we thought the mechanism of BI9564 and BI7273 in inducing apoptosis of BRD9-overexpressed gastric cancer cells should be found to explain this phenomenon.



Figure 3 Information of BI-9564 and BI-7273

A: BI9564 molecular structure; B: BI7273 molecular structure; C: Determination of IC₅₀ in AGS cells treating with BI9564; D: Determination of IC₅₀ in AGS cells treating with BI7273; E: Determination of IC₅₀ in MGC-803 cells treating with BI9564; F: Determination of IC₅₀ in MGC-803 cells treating with BI7273.

Though RNA-seq analysis and bioinformatics we found the Oxytocin pathway controlled by BRD9 inhibitors

In order to find the signaling pathway controlled by BRD9 inhibitors in gastric cancer cells, we sent the samples added BI9564 or BI7273 in AGS cells and MGC-803 cells to analysis differentially expressed genes. Though high throughput sequencing analysis the differentially expressed

genes were found (Figure 4A, Figure 4B, Figure 4C). According to volcano map we screened 22 downregulation genes and 25 upregulation genes in MGC-803 cells after inhibited BRD9 (Figure 4C); we also screened 12 downregulation genes and 11 upregulation genes in AGS cells after inhibited BRD9 (Figure 4D). On the basis of figure 4C and figure 4D, we used software, database for annotation, visualization and integrated discovery-DAVID, to analysis these changed genes and find some high score carcinogenic pathways (Figure 4E). Then we searched a large number of references, we found one of these pathways, the Oxytocin signaling pathway, may be related to BRD9 inhibitors, and its mechanism with BRD9 is remaining to be studied.



Figure 4 Analysis pathway induced by BI-9564 and BI-7273

A: MGC-803 cells' volcano map of differential gene expression; B: AGS cells' volcano map of differential gene expression; Green means downregulation genes and red means upregulation genes; C: Impact of mutation on RNA transcription in MGC-803 cells; D: Impact of mutation on RNA transcription in AGS cells; E: Signaling Pathway Analysis from KEGG.

BRD9 mediated CACNA2D4 and CALML6 expression

To find the exact genes controlled by BRD9 in the Oxytocin pathway, we applied the KEGG data base, chose some changed genes, and also verify the chose genes with RT-PCR assay. From KEGG we found that upregulated CACNA2D4 will promote CALML6 to raise, these effects will induce anti-apoptotic and inhibit oxytocin production (Figure 5A). The RT-PCR assay showed that the expression of CACNA2D4 and CALML6 were all downregulated after treated by BI9564 or BI7273 (Figure 5B and 5C), that is to say, inhibiting BRD9 can induce apoptotic and prompt secretion of oxytocin. Studies have shown that oxytocin secretion is negatively correlated with the risk of esophageal, gastric and pancreatic cancer [23-27]. Breastfeeding can induce production of oxytocin, and in this way, the risk of esophageal cancer reduced by 54% [28]. Therefore, promoting the secretion of oxytocin is conducive to inhibiting the occurrence and development of gastric cancer. From above results we speculate that BRD9 regulates the oxytocin signaling pathway in gastric cancer, and is associated with carcinogenic genes CACNA2D4 and CALML6.



Figure 5 BRD9 mediated CACNA2D4 and CALML6 expression

A: Regulation Map of Oxytocin Signaling Pathway; B: The RNA expression levels of CACNA2D4 was measured by quantitative real-time PCR in MGC-803 cells after BI9564 and BI7273 treatment; C: The

RNA expression levels of CALML6 was measured by quantitative real-time PCR in MGC-803 cells after BI9564 and BI7273 treatment.

BRD9 inhibitors enhance the sensitivity of over-expressed BRD9 gastric cancer cells to ADR and CDDP

From above results, we found BI9564 and BI7273 can inhibit CACNA2D4 and CALML6 expression in oxytocin signal pathway to induce apoptosis in over-expressed BRD9 gastric cancer cells. Then we inferred that combination BI9564 or BI7273 with ADR or CDDP would cut down the dosage of chemotherapeutic agents in curing gastric cancer, reduce the adverse effect of chemotherapeutic agents and decrease the possibility of inducing drug resistance in over-expressed BRD9 gastric cancer. To detect the infer, firstly we use MTT assay to find the IC₅₀ of ADR and CDDP in MGC-803 cells. The IC₅₀ value of ADR and CDDP in MGC-803 cells are 0.972ug and 1.889ug, respectively (Figure 6A, 6B). The viability of each dosage was shown in table 1 and 3. When we added 2.5ug/ml ADR in MGC-803 cells, the cell viability was decrease to 42.76%, but if 500nm BI9564 was added additional with 2.5ug/ml ADR the cell viability was decrease to 22.44% in MGC-803 cells (Figure 6C, Table 1), and when the dosage of BI9564 was enhanced to 1000nm and added with 2.5ug/ml ADR in MGC-803 cells the cell viability was cut down to 15.95% (Figure 6D, Table 1). On the contrary, when 5ug/ml ADR added into MGC-803 cells, the cell viability still high at 41.48% (Figure 6A, Table 1), and when the ADR dosage increased to 10g/ml the cell viability is 35.6% (Figure 6A, Table 1). The viability values of single using ADR to kill MGC-803 cells are higher than combination method, even if the dosage of ADR as high as 10g/ml. From these results, we can see that combination BI9564 with ADR in MGC-803 cells will greatly reduce the MGC-803 cell viability, and the combination medicines effect is more excellent than only treated with ADR in MGC-803 cells. Then we change the BRD9 inhibitor BI9564 to BI7273, the same trends were shown in table 2 and Figure 6E, 6F. The effects of combination BI7273 with ADR in MGC-803 cells is better than only treated with ADR. To test the university of these methods in curing over-expressed BRD9 gastric cancer patients, we changed ADR to CDDP in these experiments. From table 3 and table 4 we get the same conclusion that combination BI9564 or BI7273 with CDDP will greatly cut down the survival rate in over-expressed BRD9 gastric cancer cells (Figure 6F, 6G, 6H, 6J). These results shown that BI9564 and BI7273 can enhance the sensitivity of over-expressed BRD9 gastric cancer cells to ADR and CDDP, cut down the dosage of chemotherapeutic agents, reduce the adverse effect of chemotherapeutic agents and decrease the possibility of inducing drug resistance in over-expressed BRD9 gastric cancer.



Figure 6 MTT assays in MGC-803 cells

A: Determination of IC₅₀ in MGC-803 cells with ADR. B: Determination of IC₅₀ in MGC-803 cells with CDDP; C: Combination BI9564 (500nm) with ADR therapy in MGC-803 cells. D: Combination BI9564 (1000nm) with ADR therapy in MGC-803 cells; E: Combination BI7273 (500nm) with ADR therapy in

MGC-803 cells. F: Combination BI7273 (1000nm) with ADR therapy in MGC-803 cells; G: Combination BI9564 (500nm) with CDDP therapy in MGC-803 cells. H: Combination BI9564 (1000nm) with CDDP therapy in MGC-803 cells; I: Combination BI7273 (500nm) with CDDP therapy in MGC-803 cells. J: Combination BI7273 (1000nm) with CDDP therapy in MGC-803 cells.

Table 1 Comparison between using single ADR or combined BI9564 with ADR in MGC-803 cells

	Cell Type	2.5ug/ml ADR cell viability %	5ug/ml ADR cell viability %	10ug/ml ADR cell viability %
ADR (ug/ml)	MGC-803	42.76	41.48	35.6
500nmBI9564+ADR (ug/ml)	MGC-803	22.44	25.56	22.76
1000nmBI9564+ADR (ug/ml)	MGC-803	15.95	14.69	-

ADR, Adriamycin. Concentration gradient of ADR for MTT is 0, 2.5, 5, 10, 20, 40 µg/ml

Table 2 Comparison between using single ADR or combined BI7273 with ADR in MGC-803 cells

	Cell Type	2.5ug/ml ADR cell viability %	5ug/ml ADR cell viability %	10ug/ml ADR cell viability %
ADR (ug/ml)	MGC-803	42.76	41.48	35.6
500nmBI7273+ADR (ug/ml)	MGC-803	25.9	23.38	18.68
1000nmBI7273+ADR (ug/ml)	MGC-803	13.58	12.2	-

ADR, Adriamycin. Concentration gradient of ADR for MTT is 0, 2.5, 5, 10, 20, 40 µg/ml

Table 3 Comparison between using single CDDP or combined BI9564 with CDDP in MGC-803 cells

	Cell Type	0.5ug/ml CDDP cell viability %	1ug/ml CDDP cell viability %	2ug/ml CDDP cell viability %
CDDP (ug/ml)	MGC-803	94.19	84.81	23.91
500nmBI9564+CDDP (ug/ml)	MGC-803	19.04	16.81	14.65
1000nmBI9564+CDDP (ug/ml)	MGC-803	13.36	13.06	-

CDDP, Cisplatin. Concentration gradient of CDDP for MTT is 0, 0.5, 1, 2, 4, 8 µg/ml

Table 4 Comparison between using single CDDP or combined BI7273 with CDDP in MGC-803

	Cell Type	0.5ug/ml CDDP cell viability %	1ug/ml CDDP cell viability %	2ug/ml CDDP cell viability %
CDDP (ug/ml)	MGC-803	94.19	84.81	23.91
500nmBI7273+CDDP (ug/ml)	MGC-803	17.4	17.28	13.83
1000nmBI7273+CDDP (ug/ml)	MGC-803	13.72	12.84	-

CDDP, Cisplatin. Concentration gradient of CDDP for MTT is 0, 0.5, 1, 2, 4, 8 µg/ml

Discussion

The adverse effect of first-line chemotherapeutic drugs is a hard challenge in curing cancer patients, because most mechanisms of this type of medicine are targeting DNA and the healthy cells are unavoidable hurt. And the quality of patients' life is greatly reduced and poor prognosis or drug resistance is induced too. Some studies have shown epigenetic change are related to cancer occurrence and progression, so we inferred that considering epigenetic factor in curing cancer patients will help to alleviate these problems. BRD9 is one of the epigenetic readers in clinic, and is universal changed in a range of cancer type, and the change data in gastric cancer is 26%. Then we are very curious what's the cancerigenic mechanism will be caused by BRD9 in this kind gastric cancers, and what's the relationship between BRD9 and cancerigenic pathway. BRD9 is a subunit of SWF/SNF, and it can read acetylated histone tails for SWF/SNF binding convenience. Oncogenes expression may be induced via being bound by SWF/SNF. We infer that BRD9-overexpressed in these kinds of gastric cancer cells may lead more SWF/SNF to combine on oncogene, and active oncogene pathway. To confirm these inferences we screened some gastric cancer cells, though RT-PCR assay we chose MGC-803 cells as BRD9-overexpressed model and AGS cells as control model. Then, we found MGC-803 cells are more sensitive to BRD9 inhibitor than AGS cells. That is to say, inhibiting BRD9 in MGC-803 cells are more effective than in AGS cells. From these results, we inferred BRD9 may induce some oncogene pathway in BRD9 over-expressed cancer cells. For further finding the exact oncogenes pathway controlled by BRD9, we used RNA-seq to find the difference expression gene after adding BRD9 inhibitors. Combining the RNA-Seq results and KEGG analysis, we revealed the underlying cancerigenic mechanism, oxytocin pathway, caused by BRD9 in MGC-803 cells. For further finding the exact oncogenes targeted by BRD9, we tested some oncogenes mRNA level in oxytocin pathway after added BRD9 inhibitors, and found the gene CACNA2D4 and CALML6 are the targeted genes by BRD9. Thus, CACNA2D4 and CALML6 may serve as potential therapeutic targets in those BRD9-overexpressed gastric cancers. Then we infer that the combination of BI9564 or BI7273 with ADR or CDDP may be a good treatment for this type of gastric cancer. The MTT assays of combination medicines approved this infer. In the BRD9 over-expressed gastric cancer we can use BI9564 or BI7273 to inhibit the effect of BRD9 enhance the ADR or CDDP medicine's effect and reduce the adverse of first-line chemotherapeutic agents to BRD9-overexpressed gastric cancer patients.

Adriamycin (ADR, a chemotherapeutic drug) and cisplatin (CDDP, a chemotherapeutic drug) are first-line chemotherapeutic agents for treating solid tumors [29]. But a large number of patients induced great side effect after chemotherapy and produce successive tumor reversion and made the failure in treating cancer with ADR or CDDP [30–33]. In our study, we found BRD9 over-expressed condition in gastric cancer is universal, the clinical incidence rate is 26%. We used experiment to verify the function of BRD9 in cancer cells and found that if we use BRD9 inhibitors to treat MGC–803 cells and AGS cells the over-expressed BRD9 MGC–803 cells were more sensitive to BI9564 or BI7273 than AGS cells. To examine the potential mechanism may mediate by BRD9 in this phenomenon, we performed a screening for changed genes towards BRD9 inhibitors treatment, and with the help of analysis software DAVID, we found the BRD9 may be related with cell adhesion molecules, oxytocin signal pathway, and gastric acid secretion and so on. What is the special pathway in the BRD9 over-expressed gastric cancer controlled by BRD9? With this question we resorted to some references, some studies have shown that oxytocin secretion is negatively correlated with the risk of esophageal, gastric and pancreatic cancer [24–28], so we thought that oxytocin signal pathway may play a key role in BRD9-induced drug resistance in MGC–803 cells.

The above conclusion is based on the software prediction, the truth conclusion should be tested by experiments. Further, we examined the mRNA expression of some oncogenes in oxytocin signal pathway, then we found CACNA2D4 and CALML6 were downregulated after adding BRD9 inhibitors in MGC–803 cells. The arrangement of CACNA2D4 and CALML6 relationship with the effect was shown in figure 5A. Upregulating CACNA2D4 will promote CALML6 expression, enhanced amount of CALML6 would produce anti-apoptotic, negative regulation of oxytocin signal pathway effect. By using BRD9 inhibitors, the oncogenes expression are downregulated. Although we found the pathway controlled by BRD9, the key problem for BRD9 over-expressed gastric cancer patients is what's the effective way to cure their disease.

In order to find the possible method for curing BRD9-overexpressed gastric cancer patients, the next step we combined BRD9 inhibitor with ADR or CDDP to treat MGC–803 cells. Combination methods achieved amazing effectivity, single used 2.5ug/ml ADR achieved 57.25% mortality, ever we added the dosage to 10ug/ml ADR the mortality still at 64.4%, but when we combined 500nm BI9564 with 2.5ug/ml ADR the mortality achieved to 77.56%, and when BI9564 dosage added to 1000nm combined with 2.5ug/ml ADR the mortality achieved to 84.05%. When we change BRD9 inhibitor to BI7273 in these assays the same trend was shown in table 2. Then we change chemotherapeutic drugs to CDDP in order to verify the wide applicability of this method, as shown in table 3 and table 4, single used 0.5ug/ml CDDP only achieved 5.81% mortality, ever we added the dosage to 1ug/ml CDDP the mortality still at 15.19%, but when we combined 0.5ug/ml CDDP with 500nm BI9564 the mortality achieved to 80.96%, and when BI9564 dosage added to 1000nm combined with 0.5ug/ml CDDP the mortality achieved to 83.19%. When we change BRD9 inhibitor to BI7273 in these assays the same trend was shown in table 4. Through combining BRD9 inhibitor with chemotherapeutic drugs in over-expressed BRD9 gastric cancer cells we can kill cancer cells more easily than single used Chemotherapeutic drugs. In this way less chemotherapeutic drugs are needed in curing over-expressed BRD9 gastric cancers, less adverse effect will be induced by chemotherapeutic drugs, and to some extent suppress the appearance of drug resistance.

Our study offered one individualized drug use method based on genotype comparison to reduce chemotherapeutic side effect, and identified a new regulatory pathway BRD9/CACNA2D4 / CALML6, that contributed the induction of chemotherapeutic drugs resistance in BRD9 over-expressed gastric cancer cells. BI9564 and BI7273 are able to suppress BRD9 expression in MGC-803 cells. The molecular mechanism underlying sensitivity induced by BI9564 and BI7273 is depression of BRD9 and restraint of CACNA2D4, thus downregulating expression of CALML6 in Oxytocin signal pathway and inducing apoptosis. Based on this molecular mechanism we found an effective therapy method in curing BRD9 over-expressed gastric cancer, that is combination of BI9564 or BI7273 with ADR or CDDP will greatly enhance the drug effect of chemotherapeutic drugs cut down adverse effect of first-line chemotherapeutic agents. This study investigated the specific molecular mechanism of BRD9-mediated Oxytocin signaling pathway on BRD9 over-expressed gastric cancer, and elucidate the feasibility and effectiveness of combining BRD9 inhibitors with first-line chemotherapeutic agents in curing BRD9 over-expressed gastric cancers, providing a scientific theoretical basis on chemotherapy regimen in BRD9 over-expressed gastric cancer.

Declarations

Author contributions

Yuan Wang and Xi-Yong Yu conceived and designed the study. Yuan Wang, Xue-Yan Jiang and Guo-Dong Zheng performed experiments; Yuan Wang, Xue-Yan Jiang, Chuan-Shan Xu, Lu Liang, Zhong-Xiao Lin wrote the manuscript. All authors reviewed and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in its supplementary information files and are available from the corresponding author on reasonable request.

Compliance with ethical standards

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Conflict of interest

All authors declare that they have no conflict of interests.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

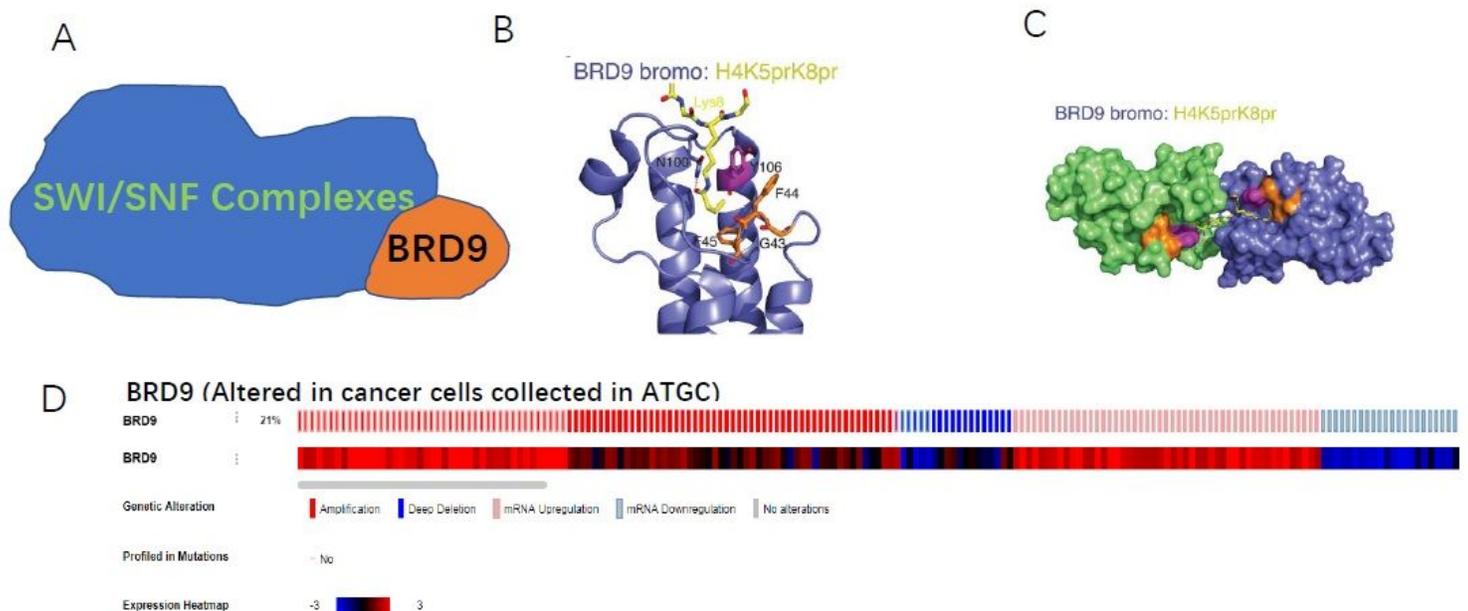


Figure 1

Information of BRD9 A: The BRD7/9 bromodomain-containing proteins are subunits in the SWI/SNF complexes; B: Coordination of H4K5prK8pr by the BRD9 bromodomain; C: Surface representation of the BRD9 bromodomain dimer in complex with H4K5prK8pr (Monomers colored in green and blue); D: Landscape of genomic aberrations in BRD9 gene in cancer cells.

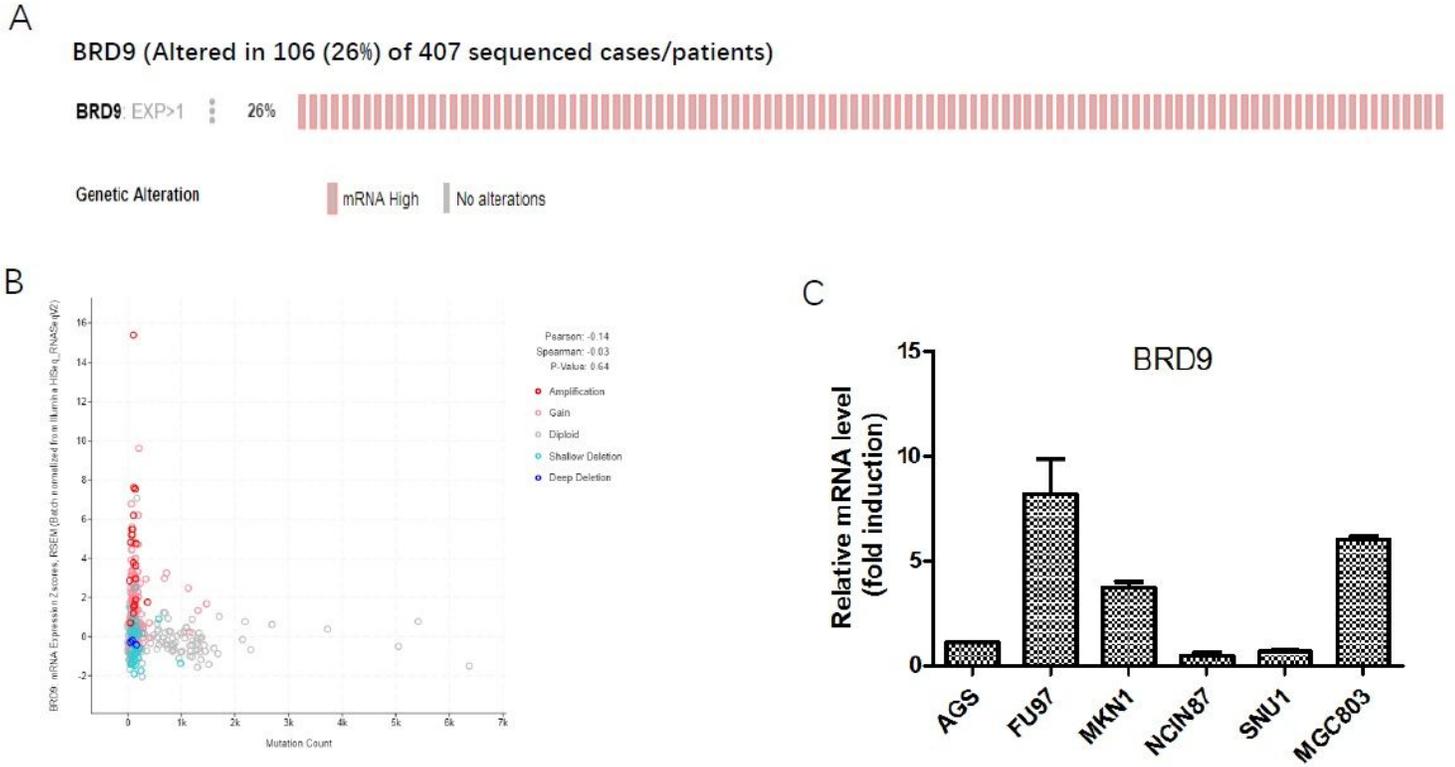


Figure 2

Expression of BRD9 in gastric cancer A: Landscape of genomic aberrations in BRD9 gene in 407 patients with gastric cancer; B: Changes of gene BRD9 in gastric cancer patient; C: Results of RT-PCR in six kinds of gastric cancer cells

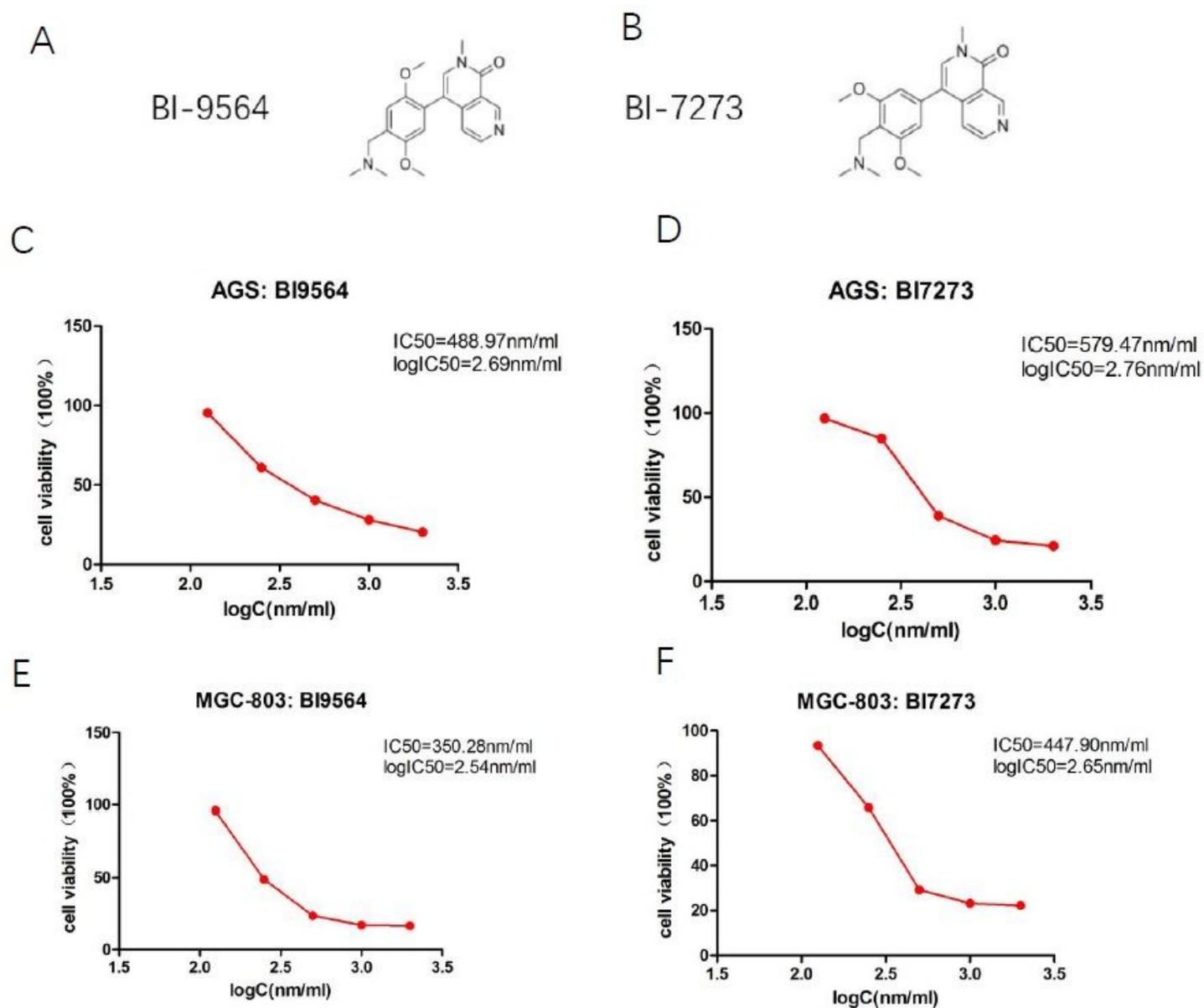


Figure 3

Information of BI-9564 and BI-7273 A: BI9564 molecular structure; B: BI7273 molecular structure; C: Determination of IC₅₀ in AGS cells treating with BI9564; D: Determination of IC₅₀ in AGS cells treating with BI7273; E: Determination of IC₅₀ in MGC-803 cells treating with BI9564; F: Determination of IC₅₀ in MGC-803 cells treating with BI7273.

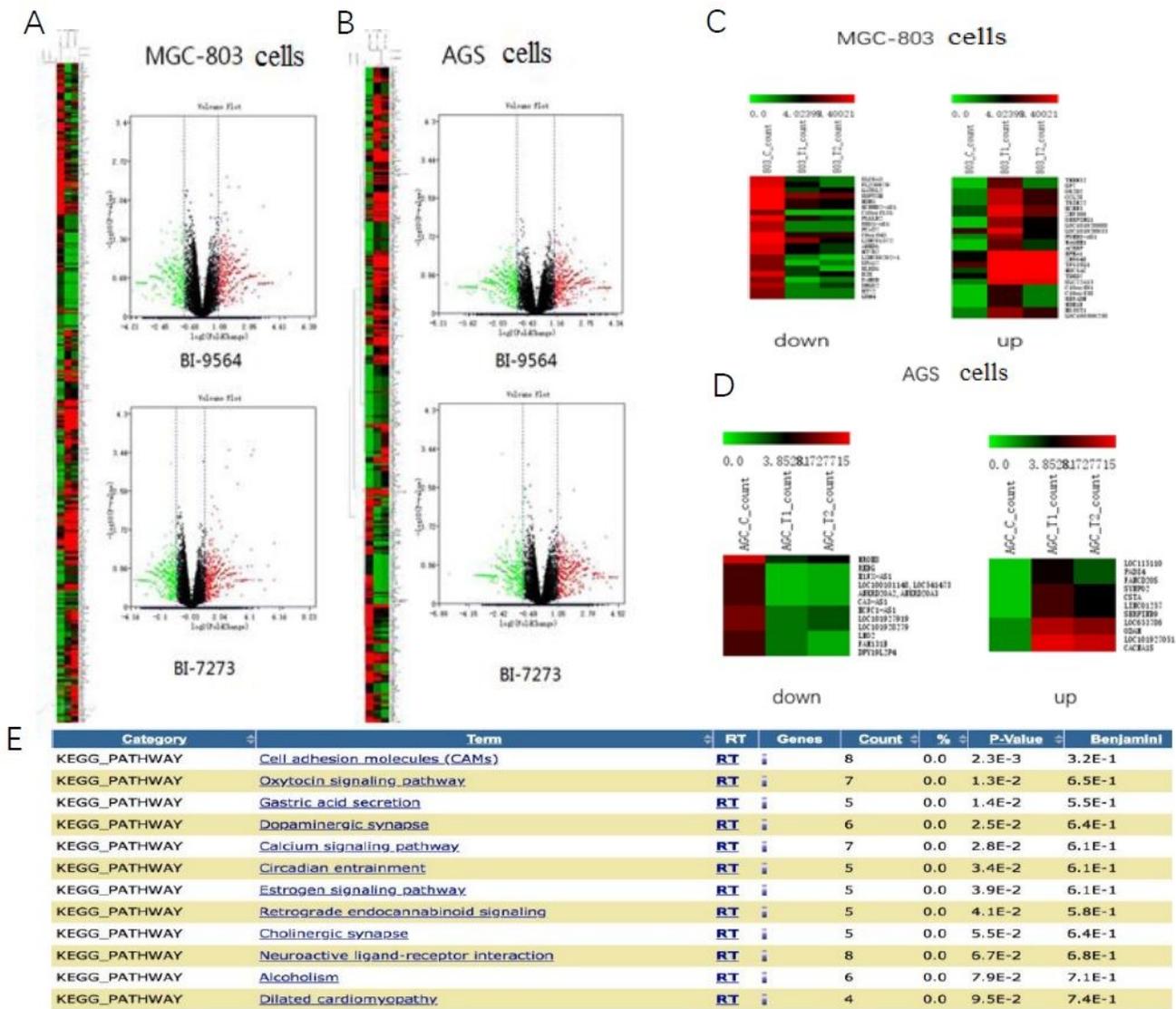


Figure 4

Analysis pathway induced by BI-9564 and BI-7273 A: MGC-803 cells' volcano map of differential gene expression; B: AGS cells' volcano map of differential gene expression; Green means downregulation genes and red means upregulation genes; C: Impact of mutation on RNA transcription in MGC-803 cells; D: Impact of mutation on RNA transcription in AGS cells; E: Signaling Pathway Analysis from KEGG.

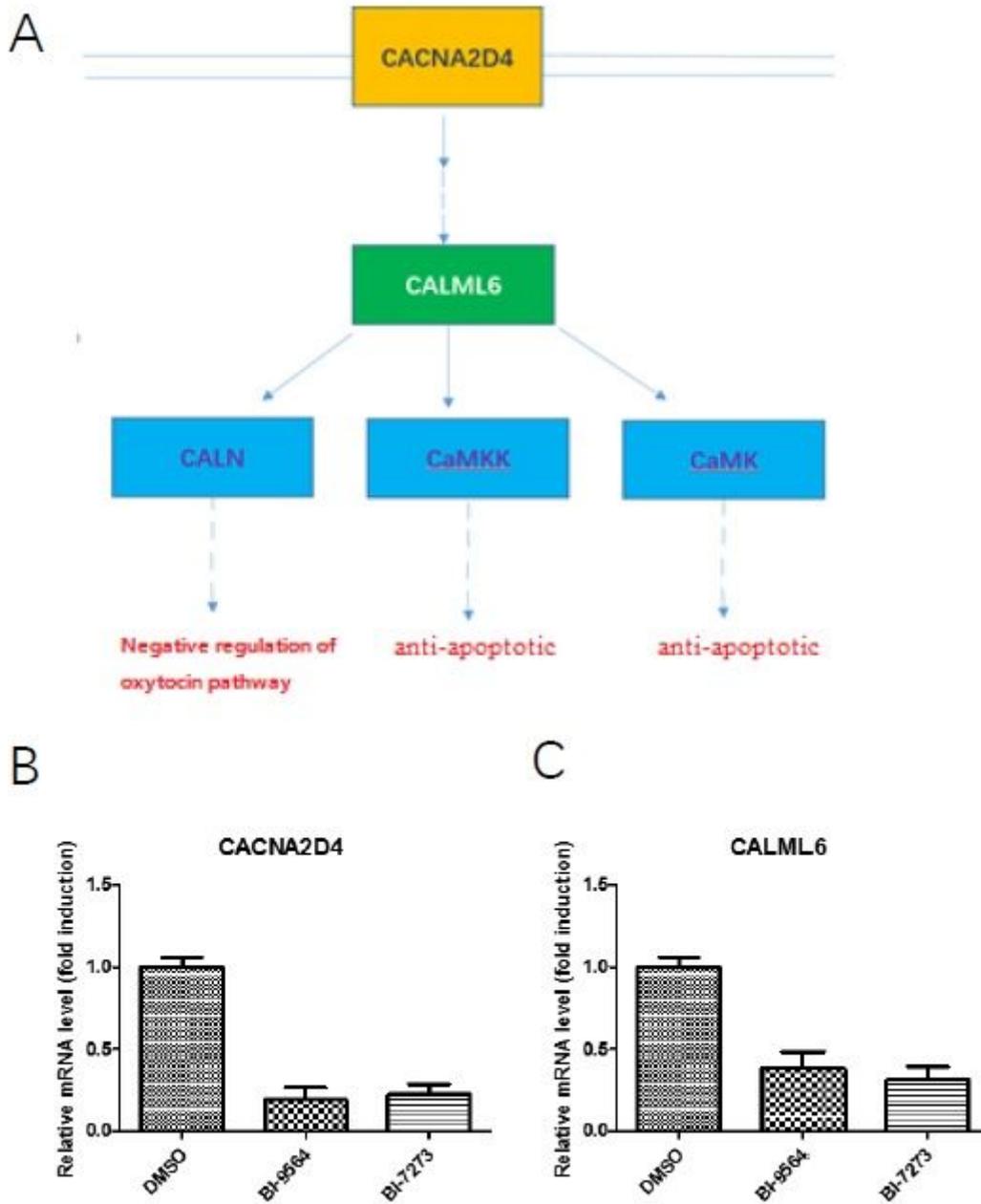


Figure 5

BRD9 mediated CACNA2D4 and CALML6 expression A: Regulation Map of Oxytocin Signaling Pathway; B: The RNA expression levels of CACNA2D4 was measured by quantitative real-time PCR in MGC-803 cells after BI9564 and BI7273 treatment; C: The RNA expression levels of CALML6 was measured by quantitative real-time PCR in MGC-803 cells after BI9564 and BI7273 treatment.

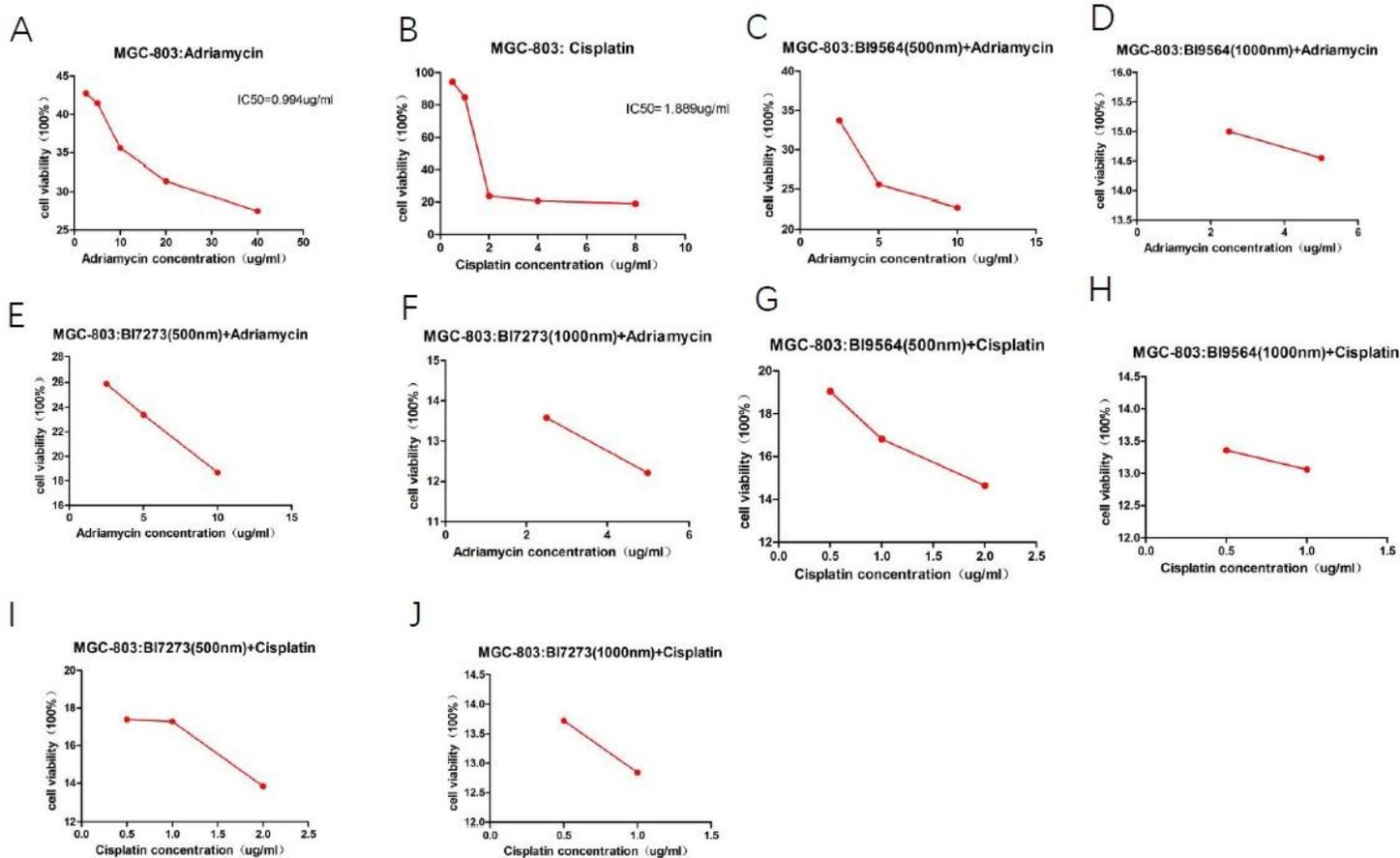


Figure 6

MTT assays in MGC-803 cells A: Determination of IC50 in MGC-803 cells with ADR. B: Determination of IC50 in MGC-803 cells with CDDP; C: Combination BI9564 (500nm) with ADR therapy in MGC-803 cells. D: Combination BI9564 (1000nm) with ADR therapy in MGC-803 cells; E: Combination BI7273 (500nm) with ADR therapy in MGC-803 cells. F: Combination BI7273 (1000nm) with ADR therapy in MGC-803 cells; G: Combination BI9564 (500nm) with CDDP therapy in MGC-803 cells. H: Combination BI9564 (1000nm) with CDDP therapy in MGC-803 cells; I: Combination BI7273 (500nm) with CDDP therapy in MGC-803 cells. J: Combination BI7273 (1000nm) with CDDP therapy in MGC-803 cells.