

Original Article

Cardiac glycosides inhibit proliferation and induce apoptosis of human hematological malignant cells

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Abstract: Cardiac glycosides have been used to treat heart diseases for centuries. Recently, the emerging role of these compounds in cancer treatment is being noticed. Mounting evidences suggest that these steroid-like compounds have both anti-proliferation and pro-apoptosis effects on different human solid tumor cells. Our study mainly focuses on hematological malignant diseases. We took 19 different kind of hematological cells as research object. By calculating the IC₅₀ and apoptosis rate of each cell, we discovered that, ouabain, digitoxin, and digoxin all had growth inhibition and apoptosis induction effect with human hematological malignant cells in a time dependent manner, while found no suppressing effect on cell viability of human bone marrow mononuclear cells from people with no hematological malignant diseases. Even within the malignant cells, distinct cell types showed diversities of drug sensitivity. Cells of lymphocytic series and plasmacyte series such as ALL, MM and Burkitt's lymphoma got higher susceptibility compared to myelocytic cells. The efficacy and safety that cardiac glycosides possessed make them new type of potential targeted anti-tumor agents.

Keywords: Cardiac glycosides, proliferation, apoptosis, hematological malignant diseases

Introduction

Cardiac glycosides, a serial of steroid derivatives, are highly specific Na, K-ATPase ligand that can bind to the catalytic α -subunit of this membrane protein with inositol 1,4,5-trisphosphate receptors (IP3R) [1, 2]. The use of these glycosides may date back to over 1500 years [3], whose most common application in modern time is mainly focused on treatment of congestive heart failure and some types of cardiac arrhythmias.

Despite of curing cardiac diseases, cardiac glycosides also found their roles in cancer treatment. In 1970s, Stenkvist [3, 4] noticed an outstanding association between digitalis taking and better prognosis of breast cancer, suggesting a potential anticancer effect of this cardiac glycoside. Since then, increasing number of researches were discovering the anti-proliferation and pro-apoptosis effects of cardiac glycosides on different human malignant tumor cells, including lung cancer, breast cancer and prostate carcinoma and so on [5-12]. While

most studies were focusing on curing solid tumors, few hematological malignant diseases have been mentioned. To examine whether cardiac glycosides would interact with hematological malignant cells on proliferation and apoptosis, we took 18 different kind of hematological malignant cells as research object. By calculating the IC₅₀ and apoptosis rate of each malignant cell and repeat the experiments on human bone marrow mononuclear cells, we discovered the drug effective concentrations and made the efficacy and safety evaluation of these glycosides.

Materials and methods

Cells

Human malignant hematological cell lines were purchased from Shanghai Institute Cell Bank. Human bone marrow mononuclear cells (BM-MNCs) were separated with Ficoll-Hypaque [13] from patients who were confirmed to have no hematological malignant diseases. Primary human malignant hematological cells were col-

The inhibition effect of cardiac glycosides on hematological malignant cells

lected from patients who were diagnosed with hematological diseases. Cells were cultured in RPMI1640 medium (GIBCO/Invitrogen, USA) containing 10% FBS, in 5% CO₂ at 37°C. Cell No.1 to No.12: KM3: multiple myeloma cell line, CZ: multiple myeloma cell line, MM1-S: multiple myeloma cell line, MM1-R: multiple myeloma cell line, RPMI 8226: multiple myeloma cell line, Mino: mantle cell lymphoma cell line, Jeko1: mantle cell lymphoma cell line, Raji: human Burkitt's lymphoma cell line, Jurkat: acute T cell leukemia cell line, NB4: acute promyelocytic leukemia cell line, Kasumi-1: acute myeloblastic leukemia cell line, BM-MNCs: human bone marrow mononuclear cells.

All patients provided informed consent for genetic analysis based on the Declaration of Helsinki in accordance with the Ethics Committee of The Affiliated Drum Tower Hospital of Nanjing University Medical School.

Chemicals

All chemicals were obtained from Sigma (USA) unless otherwise stated. Chemicals were used in the following concentrations in all experiments: ouabain 20 nM, ouabain 50 nM, digitoxin 20 ng/ml, digoxin 30 ng/ml (Biomol International, UK). The concentrations were determined by preexperiment, which found these cardiac glycosides could inhibit cell growth and induce apoptosis in a dose-dependent manner between 20 and 100 nM.

Measurements of cell viability and proliferation

Malignant cells and BM-MNCs (5×10^3 - 1×10^4 /well) were plated in 0.1 ml medium (RPMI-1640 with 10% FBS) in 96-well plates. On culture day 2, 1 μ l different concentrations of cardiac glycosides were added into each well. After 24 h, 48 h, 72 h of incubation, cell viability was measured by the Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's protocol. The optical densities at 450 nm were measured using a 96-well multi-scanner autoreader and the cell viability was calculated.

Detection and quantification of apoptotic cells

Malignant cells and BM-MNCs (5×10^4 - 1×10^5 /well) were plated in 1 ml medium (RPMI-1640 with 10% FBS) in 24-well plates. On culture day 2, cells were treated with ouabain (50 nM) for

48 h. For the analysis of apoptotic cells, the sample was prepared using an Annexin-V-FLOUS Staining Kit (Roche, Germany) according to the manufacturer's instructions. The Annexin-V/PI stained cells were then analyzed by flow cytometry.

Statistical analysis

Data are presented as mean of at least three independent experiments \pm standard deviation of mean. Significance was estimated by using oneway ANOVA tests, as further detailed in figure legends. *P*-values < 0.05 were considered as significant.

Results

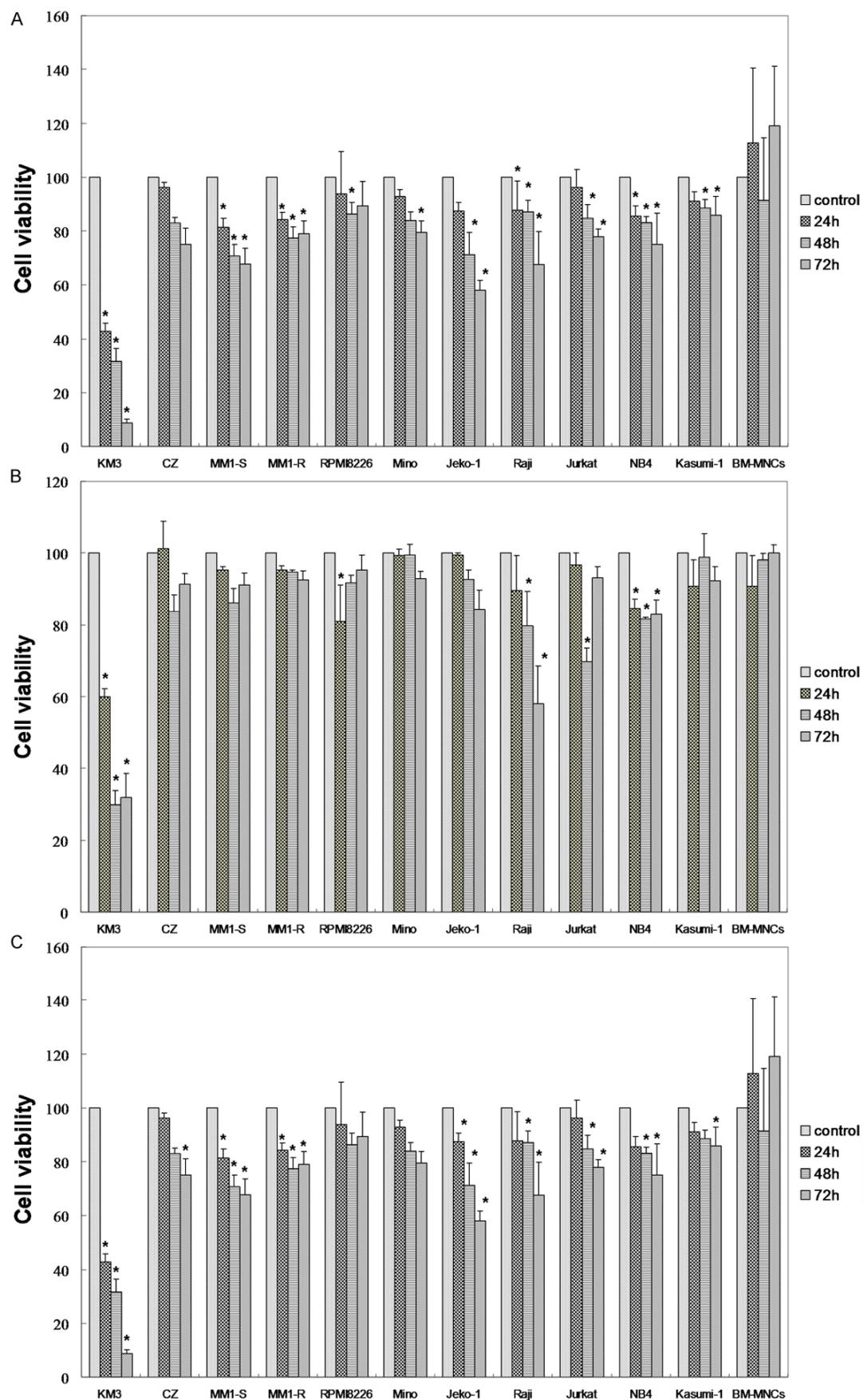
Growth inhibition effect of cardiac glycosides

After incubated for 24 h, 48 h, 72 h in the presence or the absence (control) of the cardiac glycosides (ouabain 20 nM, digitoxin 30 ng/ml, digoxin 30 ng/ml), a time-dependent growth inhibition of human hematological malignant cells was seen, while no significantly changes in BM-MNCs were found (**Figure 1**) (Note: The drug concentrations used in this study were based on our previous experiment, in which we tried different drug concentrations on each cell respectively, and a dose-dependent inhibition effect was discovered, although not displayed in this study). IC₅₀ values in 48 h of both malignant cells and BM-MNCs for ouabain, digitoxin and digoxin were determined respectively (**Table 1**). As data shown, cells of lymphocytic series and plasmacyte series such as ALL, Burkitt's lymphoma and MM got lower IC₅₀ values compared with myelocytic cells, (**Table 1; Figure 2**). For example, myeloma cell line KM3, with the lowest IC₅₀ values in the 10-30 nM concentration ranges, was the most susceptible to cardiac glycosides, while the Burkitt's lymphoma cells also showed hypersensitive to cardiac glycosides, whose IC₅₀ values in the 40-70 nM concentration ranges. The primary malignant cells of lymphocytic series and plasmacyte series such as ALL, Burkitt's lymphoma and MM got IC₅₀ values range from 50-100 nM, while myelocytic cells like AML got IC₅₀ values range from 90-300 nM.

Apoptosis induction effect of ouabain

The effect of ouabain on apoptosis was studied in both malignant cells and BM-MNCs, as

The inhibition effect of cardiac glycosides on hematological malignant cells



The inhibition effect of cardiac glycosides on hematological malignant cells

Figure 1. Cardiac glycosides suppress cell viability of malignant cells, but induce no significantly changes in BM-MNCs. All cell lines were incubated for 24 h, 48 h, 72 h in the presence or the absence (control) of cardiac glycosides. A. Ouabain (20 nM) might inhibit cell proliferation of KM3, MM1-S, MM1-R, Jeko-1, Raji, Jurkat NB4 and Kasumi-1 cell lines detected by CCK-8 assay; B. Digitoxin (30 ng/ml) might inhibit the growth of KM3, Raji, NB4 cell lines; C. Digoxin (30 ng/ml) might repress cell proliferation of KM3, MM1-S, MM1-R, Jeko-1, Raji, Jurkat and NB4 cell lines, with only slightly changes in BM-MNCs. The inhibition of the overall viability of cells was assessed with the Cell Counting Kit-8 (CCK-8) assay. Values are mean \pm s.d; * P <0.05.

Table 1. The IC₅₀ values of cells for ouabain, digitoxin and digoxin

		Ouabain	Digitoxin	Digoxin
1	KM3	9.49 \pm 1.15	12.85 \pm 2.08	28.14 \pm 3.68
2	CZ	41.55 \pm 6.55	62.62 \pm 3.21	835.37 \pm 629.2
3	MM1-S	34.94 \pm 3.57	100.28 \pm 14.89	73.16 \pm 21.95
4	MM1-R	41.55 \pm 6.55	90.69 \pm 4.91	29.32 \pm 8.95
5	RPMI 8226	60.26 \pm 12.19	141.44 \pm 68.21	104.36 \pm 11.90
6	Mino	36.83 \pm 2.99	90.09 \pm 0.16	1291.36 \pm 851.99
7	Jeko-1	37.79 \pm 10.23	80.89 \pm 1.84	47.79 \pm 30.95
8	Raji	59.99 \pm 9.17	42.62 \pm 4.26	68.77 \pm 7.69
9	Jurkat	72.07 \pm 22.92	101.32 \pm 9.68	73.89 \pm 29.55
10	NB4	65.60 \pm 8.29	80.34 \pm 14.07	92.47 \pm 25.00
11	Kasumi-1	87.77 \pm 15.45	90.62 \pm 30.31	115.11 \pm 56.38
12	BM-MNCs	434.13 \pm 229.35	388.96 \pm 140.98	467.59 \pm 111.66
13	MM	70.26 \pm 36.67	64.59 \pm 21.51	74.99 \pm 30.74
14	Burkitt's lymphoma	57.99 \pm 28.19	56.67 \pm 24.78	81.35 \pm 32.44
15	ALL-T	99.49 \pm 49.80	105.76 \pm 50.50	100.41 \pm 41.86
16	ALL-B	76.50 \pm 44.39	89.45 \pm 34.80	90.47 \pm 45.55
17	AML-M1	124.53 \pm 54.33	197.31 \pm 36.53	207.48 \pm 49.41
18	AML-M3	270.03 \pm 29.81	307.79 \pm 29.99	276.99 \pm 31.45
19	AML-M5	106.84 \pm 31.64	94.16 \pm 40.02	137.22 \pm 47.68

Different cells were treated with cardiac glycosides for 48 h, the IC₅₀ values indicate the concentrations of cardiac glycosides that caused 50% inhibition of cell proliferation. No.1 to No.11: human malignant hematological cell lines; No.12: human bone marrow mononuclear cells (BM-MNCs); No. 13 to No.19: primary human malignant hematological cells collected from patients.

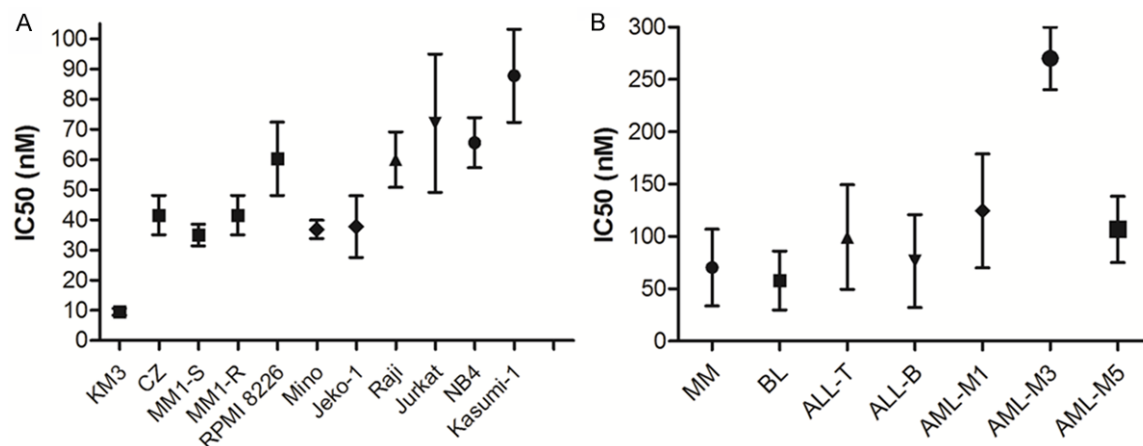


Figure 2. Cells of lymphocytic series and plasmacyte series got lower IC₅₀ values compared with myelocytic cells. All cells were incubated for 48 h in the presence or the absence (control) of ouabain. A. The myeloma cell lines like KM3, CZ, MM1-S, MM1-R and RPMI 8226 got IC₅₀ values range from 10-60 nM. The lymphocytic cell lines like Mino, Jeko-1, Raji and Jurkat got IC₅₀ values range from 30-70 nM. The myelocytic cell lines got higher IC₅₀ values that range from 60-70 nM; B. The primary malignant cells of lymphocytic series and plasmacyte series such as ALL, Burkitt's lymphoma (BL) and MM got IC₅₀ values range from 50-100 nM. The myelocytic cells like AML got IC₅₀ values range from 90-300 nM.

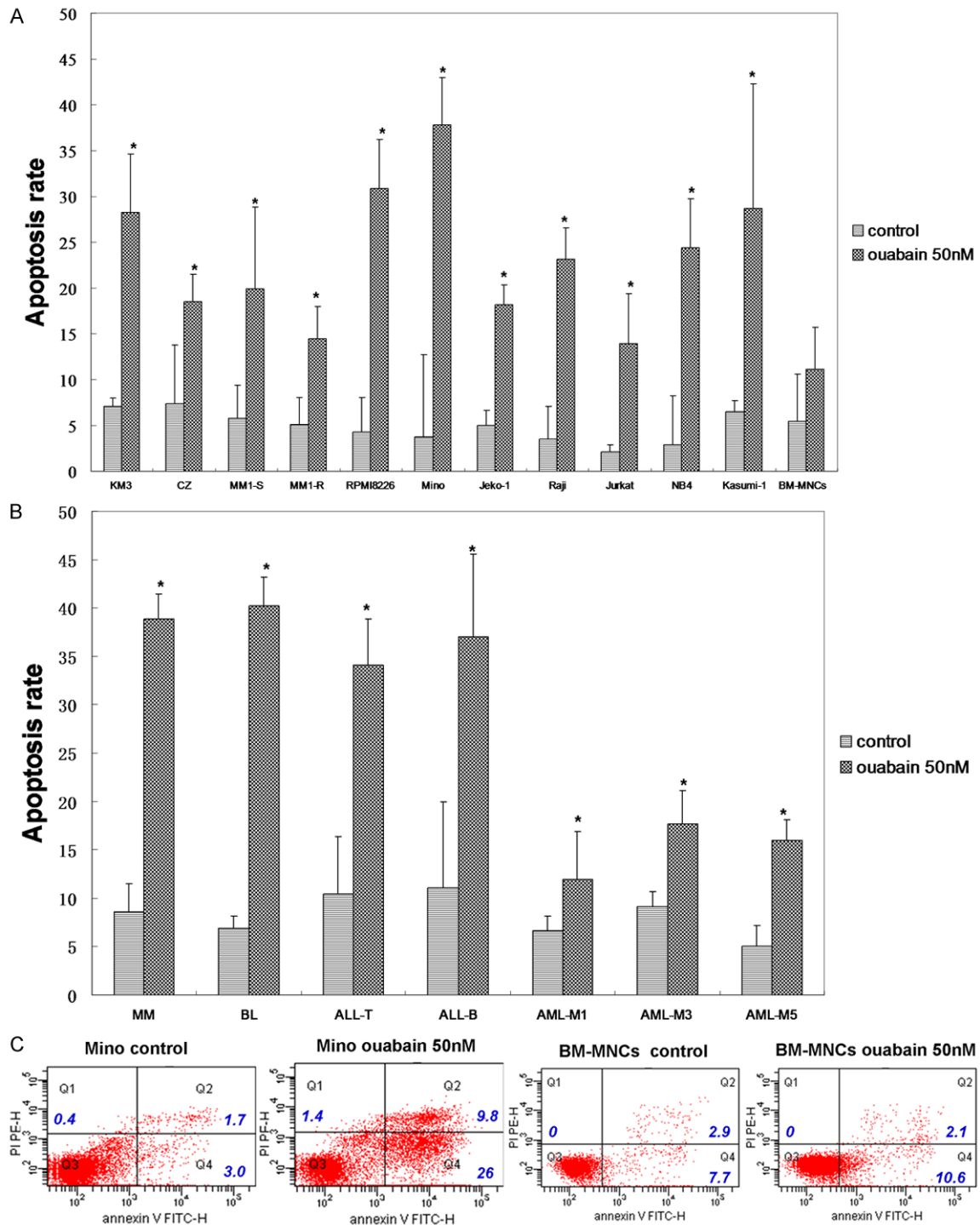


Figure 3. Ouabain induces apoptosis of human malignant hematological cells. Both malignant cells and BM-MNCs were incubated with ouabain 50 nM for 48 h. A. Ouabain (50 nM) induced marked apoptosis of human malignant hematological cell lines detected by Annexin-V and PI flow Cytometry Assay Kits; B. Ouabain (50 nM) also significantly increased the apoptosis rate of primary human malignant hematological cells. Cells from Burkitt's lymphoma (BL), MM and ALL got higher apoptosis rate compared with AML cells. The apoptosis induction effect of ouabain was detected by Annexin-V and PI flow Cytometry Assay Kits. Values are mean \pm s.d.; * $P < 0.05$; C. Both Mino cell line and BM-MNCs were incubated with ouabain 50 nM for 48 h. Cells were stained with Annexin V and PI and analyzed by flow cytometry. Cell population in each indicated quadrant is numerically depicted.

described in Materials and Methods. As data shown, ouabain 50 nM can induce apoptosis of malignant cells, both cell lines or human primary cells, while induce no significant changes in BM-MNCs (**Figure 3**). We take one of the most sensitive cell lines Mino as an example to show the high apoptosis rate (both early and late apoptosis) in **Figure 3C**, compared with BM-MNCs. Likewise, cells of lymphocytic series such as ALL, MM and Burkitt's lymphoma got higher susceptibility compared to myelocytic cells.

Discussion

Recent years, studies about the anti-cancer effect of cardiac glycosides have been accumulated. Some researches have reported the depress effect of cardiac glycosides on hematological malignant diseases. For example, Vermes discovered the ALL-T cell line Jurkat and the Burkitt's lymphoma cell line Daudi were both sensitive to digitalis [14]. Cuozzo's data outlined that cardenolides could induce cell cycle impairment of U937 cells (monocytic leukemia) [15]. But each data only taken individual cell line as research objects, and there is no evidence suggests which type of cancer cells is more sensitive to cardiac glycosides. In order to make comparisons between distinct cell types, we selected 19 different types of cells, which comprised 11 malignant hematological cell lines purchased from cell bank, 7 primary malignant hematological cells collected from patients, and the human bone marrow mononuclear cells from people without hematological malignant diseases. These cells compose most of the hematological malignancies, including multiple myeloma, mantle cell lymphoma, Burkitt's lymphoma, acute lymphocytic leukemia, and acute myelocytic leukemia. To our knowledge, this is the first study that made analysis of so many different cell types that cover the major parts of hematological diseases.

Our results indicate that, diverse cells types from plasmacyte series, lymphocytic series and myelocytic series were all sensitive to cardiac glycosides. It is worth mentioning that we initially discovered the Jeko-1 and Mino cell lines could be inhibited by cardiac glycosides, which is the first time to show the suppressing effect on mantle cell lymphoma, as no currently available data have reported similar results yet. Another point worth emphasizing is that, the

primary malignant cells that we collected from patients also turned to be cardiac glycosides sensitive in vitro, similar as cell lines purchased from cell bank. Compared with the readymade cell lines, primary cells from patients got more close characteristics of tumor cells in vivo. This may indicate favorable results in the future clinical trials of these cardiac glycosides.

The IC50 values in our study ranged from 10-300 nM for 48 h, which were similar with Cuozzo's data [15]. In addition, existing researches revealed that IC50 values of cardiac glycosides acting on solid tumors were also in the same concentration ranges. Kulkarni [10] proved that digitoxin can reduce cell vitality of lung cancer cells at a concentration of 50 nM. Delebinski [16] recently reported ouabain and digitoxin were able to inhibit proliferation of osteosarcoma cells within a range of 0.1-0.15 μ M for 24 h. Yang's team [17] showed that after incubating for 24 h, digitoxin, digoxin and ouabain were cytotoxic to HeLa cells, with threshold concentrations in the 50-100 nM concentration ranges. The above conclusions suggest that these glycosides may have similar anticancer effect on both hematological malignancies and solid tumors. As numbers of clinical trials of cardiac glycosides treating solid tumors are in progress and have shown positive results [9], clinical application on hematological malignancies can also be taken into consideration.

Moreover, this study discovered that even within the hematological malignant cells, distinct cell types showed diversities of drug sensitivity. As data shown, the primary malignant cells of lymphocytic series and plasmacyte series such as ALL, Burkitt's lymphoma and MM got lower IC50 values compared with myelocytic cells (**Table 1**; **Figure 2**). The similar trend was seen in malignant cell lines (**Table 1**; **Figure 2**). On the above basis, we speculate there may be some molecular targets that made plasmacyte and lymphocytic cells more sensitive to cardiac glycosides compared with myelocytic cells, as the anticancer mechanism is still not quite clear yet.

As many existing studies have announced the drug efficacy of cardiac glycosides in cancer treatment, few have mentioned about the medication safety. In fact, it is vital to the future use of cardiac glycosides to prove if they could

inhibit tumor cell proliferation in a low dose with an acceptable toxicity. According to our research, these glycosides may affect the apoptosis and proliferation of malignant cells, while no suppressing effect on cell viability of normal BM-MNCs was found, when treated with the same cardenolides simultaneously. This provides reliable evidence of the drug safety in vitro, and suggests that cardiac glycosides may be a new type of targeted anti-tumor agents.

Being a specific ligand of Na,K-ATPase, cardiac glycosides can dose-dependently inhibit the activity of Na,K-ATPase [18], which in addition to the role in the maintenance of Na⁺ and K⁺ gradients across the cell membrane, also act as a signal transducer [1, 19]. It is rational for us to assume that the anti-cancer effect of these steroid derivatives might be associated with the Na,K-ATPase induced signaling ways. Juan Li [1, 2, 19] believe that the interaction between ouabain-bound Na,K-ATPase and the 1,4,5-trisphosphate receptor (IP3R) can modulate the activity of the transcription factor NF-κB, which is a pleiotropic regulator of cell proliferation and apoptosis. Our team also discovered that cardiac glycosides could down regulated the protein level of c-myc in Burkitt's lymphoma cells, leading to a decrease of NF-κB level (the results not published yet). These special genes and factors that cardiac glycosides target to might partially explain the potential mechanisms for these Na,K-ATPase inhibitors as novel anti-cancer agents.

In conclusion, this investigation shows that the cardiac glycosides had growth inhibition and apoptosis induction effect of human hematological malignant cells, including ready-made cell lines and primary cells, while found no suppressing effect on cell viability of BM-MNCs from healthy people. Malignant cells of lymphocytic series and plasmacyte series got higher susceptibility compared to myelocytic cells. To further prove the efficacy and safety of these glycosides both in vitro and vivo, as well as to better understand the complex cell signal transduction mechanisms involved in, more researches still needs to be carried out.

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Disclosure of conflict of interest

None.

Authors' contribution

Jian Ouyang: provided the conception and design of the study, analysis and interpretation of data, revised the article critically for important intellectual content, and final approval of the version to be submitted; Yueyi Xu and Juan Li: acquisition of data, supplied the analysis and interpretation of data, drafting the article; Bing Chen and Min Zhou: supplied and acquisition of data, was responsible for the article critically for important intellectual content; Yi Zeng and Qian Zhang: supplied and acquisition of data; Yanting Guo and Jinhao Chen: provided artwork help, proof reading the article.

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