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A cell-based screen for anticancer activity of 13 pyrazolone derivatives

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[Abstract] Background and Objective: Pyrazolone derivatives were reported to have a potent cytotoxicity against some tumor cells. In the present study, we evaluated the cytotoxic activity of a series of pyrazolone derivatives against four human tumor cell lines including HepG2, OVCAR3, KB, and multidrug resistance (MDR) KBv200 cell lines *in vitro* and *in vivo*. Additionally, the structure-activity relationships of these compounds were discussed. **Methods:** To analyze the antiproliferative potential of the synthesized compounds against several human tumor cell lines, the 50% inhibitory concentration (IC_{50}) values were determined by MTT assay. Besides, the KBv200 cell xenograft experimental model was established and the sensitivity to the pyrazolone compounds was compared between drug-sensitive parental KB cells and MDR KBv200 cells. **Results:** Of 13 compounds screened, compound 9 presented remarkable anticancer effects, of which IC_{50} values were (3.24 ± 0.28), (2.58 ± 0.61), (3.81 ± 0.02), and (3.45 ± 0.03) μg/mL in HepG2, OVCAR3, KB and MDR KBv200 cells, respectively ($P > 0.05$). Furthermore, compound 9 effectively inhibited tumor growth of KBv200 cell xenografts *in vivo*, the inhibition ratio was 25.37%, 38.43%, and 47.50% for 1.5 mg/kg, 3 mg/kg, and 6 mg/kg of compound 9 groups, respectively. **Conclusion:** Compound 9 was the most promising antitumor agent in this study.

Key words: Pyrazolone derivative, multidrug resistance, cytotoxicity

Cancer is a major public health problem all over the world because of its significantly high rates in morbidity and mortality. Conventional cancer chemotherapy is seriously limited by tumor cells exhibiting multidrug resistance (MDR), caused by the overexpression of integral membrane transporters, such as P-gp and MDR-associated proteins (MRPs) which decrease the drug accumulation and cell death. The classic resistance to cytotoxic drugs has most often been linked to overexpression of P-glycoprotein (P-gp)[1]. P-gp acts as an ATP-dependent multidrug efflux pump transporting a broad range of drugs across membrane out of cytosol, therefore P-gp reduces intracellular bioavailability and toxicity of these drugs[2]. To date, the strategies aimed at reversing MDR have principally focused on inhibition or modulation of P-gp activity. Various MDR reversal agents have been identified and some are undergoing clinical trials, but currently none is in clinical use. Therefore, developing novel anticancer drugs efficient to MDR cells is another notable strategy for overcoming MDR.

Recently, an intense search for new chemical structures beneficial to designing antitumor drugs has been sparked and pyrazolone-base derivatives have received an extensive attention. Pyrazolone, as a prominent structural motif, is found in numerous pharmaceutically active compounds. Due to the easy preparation and rich biological activity, pyrazolone framework plays an essential role and represents an interesting template for combinatorial and medicinal chemistry. Indeed, pyrazolone-based derivatives have shown several biological activities as seen in COX-2[3], p38 MAP kinase[4], CDK2/Cyclin A inhibitors[5], telomerase inhibitor[6], antimicrobial[7] and antiviral[8]. In the search for anticancer agents, a certain number of pyrazolone compounds exhibited promising antiproliferative properties. Some studies have suggested that pyrazolone compounds had a potent cytotoxicity against HL-60 cells[9] and human lung cancer cells[10] *in vitro*. Despite the existence of some reports on their antitumor activity, literatures related to the antiproliferative effects of pyrazolone derivatives on parental

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drug-sensitive KB cells and MDR KBv200 cells either *in vitro* or *in vivo* were greatly limited.

In our study, we investigated the antitumor activity of 13 novel pyrazolone derivatives against four human tumor cells *in vitro* and explored their structure-activity relationships. Furthermore, the effect of compound 9 on tumor growth of KBv200 cell xenografts *in vivo* was also presented.

Materials and Methods

Chemicals and reagents

3-(4, 5-Dimethyl-2-thiazolyl) 2, 5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. All tissue culture supplies were purchased from Gibco-BRL Co. All the compounds with a purity of >95% were synthesized by Institute of Applied Chemistry, Xinjiang University. The basic structure of the compounds (Figure 1A), the chemical structure of compounds (Figure 1B) and the analytical and physical data of compounds (Table 1) are shown below.

Cell lines and cell culture

Human epidermoid carcinoma cell lines KB and KBv200 were used in this study. KBv200 cells, a classic multidrug-resistant cell line expressing high levels of P-gp, were induced from drug-sensitive parental KB cells by stepwise exposure to increasing doses of vincristine (VCR) and ethylmethane sulfonate (EMS) mutagenesis. Compared with the KB cell line, the KBv200 cell line was about 100-fold more resistant to VCR [11]. Cancer cell lines, including a human liver carcinoma cell line HepG2 and a human ovarian carcinoma cell line OVCAR3, obtained from the Chinese Academy of Medical Sciences (Beijing, China), were also used in this study. All the cell lines were maintained in RPMI-1640 with 10% fetal bovine serum (FBS) and cultured in a humidified atmosphere of 5% CO_2 at 37℃.

A nimals and establishment of xenograft model

Athymic nude mice (BALB/c-nu-nu), male or female (5–6 weeks old, 24–26 g), which were obtained from the Center of Experimental Animal, Sun Yat-sen University of Medical Sciences, were used for xenograft model in this study. The animals were maintained and reared with sterilized food and water.

To further evaluate the effect of effective compounds on MDR KBv200 cell xenografts *in vivo* and to extend our studies, the KBv200 cell xenograft experimental model was established in athymic nude mice (BALB/c-nu-nu) as described by Fu *et al*.[12].

In detail, transplantable KBv200 cells was harvested, then suspended at a concentration of 1×10^7 cells/mL and implanted into mice for chemotherapeutic studies. Mice received a subcutaneous (s.c.) injection of 2×10^6 cells per inoculation under the right armpits. When mean diameter of tumors was approximately 0.5 cm, animals were randomized into five groups including control group (5% Tween 80 in saline, 10 mL/kg), cyclophosphamide (CTX) alone group (100 mg/kg, intraperitoneally, once every 5 says for 10 days), and three compound alone groups (1.5, 3, and 6 mg/ kg respectively, intraperitoneally, once every day for 10 days). The weight of animals and the volume of the xenografts were measured every 2 days. Tumor volume was measured in two perpendicular diameters (A and B). Tumor volume (V) was estimated according to the formula:

$$V = \frac{\pi}{6} \left(\frac{A + B}{2}\right)^3$$

The curve of tumor growth was drawn according to

Table 1 Analytical and physical data of pyrazolone compounds

Compound	Abbreviation	Molecular formula
1-phenyl-3-methyl-4-(2-hydroxylbenzylidenehydrazine)-p-methylbenzylidene-5-pyrazolone	PMPTP-SAH	$C_{25}H_{22}N_4O_2$
1,3-diphenyl-4-(2-hydroxylbenzylidenehydrazine)-ethidine-5-pyrazolone	PPAP-SAH	$C_{24}H_{20}N_4O_2$
1-Phenyl-3-methyl-4-propionyl-5-pryazolone semicarbazone	PMPP-SC	$C_{14}H_{16}N_5O_2$
1,3-diphenyl-4-(p-nitrobenzoylhydrazide)-ethidine-5-pyrazolone	PPAP-PNBH	$C_{24}H_{18}N_5O_4$
1,3-diphenyl-4-(2-ethylamino-6H-1,3,4-thiadiazine-5-ylene)-5-pyrazolone	PPCP-ETSC	$C_{20}H_{19}N_5OS$
1-Phenyl-3-methyl-4-(ethylaminoformylhydrazide)-p-methylbenzylidene-5-pyrazolone	PMPTP-ETSC	$C_{21}H_{23}N_5O_2$
1-phenyl-3-methyl-4-(methylaminoformylhydrazide)-p-methylbenzylidene-5-pyrazolone	PMPTP-MTSC	$C_{25}H_{21}N_4O_2$
1,3-diphenyl-4-propionyl-5-pyrazolone semicarbazone	PPPP-SC	$C_{19}H_{18}N_5O_2$
[CuL(EtOH)]1-Phenyl-3-methyl-4-(salicyclidenehydrazide)-propylidene-5-pyrazolone	Cu(II) PMPP-SAL	$C_{20}H_{20}N_4O_3Cu(II)$
[CuL(EtOH)]1-Phenyl-3-methyl-4-(2-hydroxylbenzylidenehydrazine)-propylidene-5-pyrazolone	Cu(II) PMPP-SAH	$C_{20}H_{20}N_4O_2Cu(II)$
1-Phenyl-3-methyl-4-(2-hydroxylbenzoylhydrazide)-propylidene-5-pyrazolone	PMPP-SAL	$C_{20}H_{20}N_4O_3$
1-Phenyl-3-methyl-4-(2-hydroxylbenzylidenehydrazine)-propylidene-5-pyrazolone	PMPP-SAH	$C_{20}H_{20}N_4O_2$
1-Phenyl-3-methyl-4-(methylaminoformylhydrazide)-propylidene-5-pyrazolone	PMPP-MTSC	$C_{15}H_{19}N_5O_2$

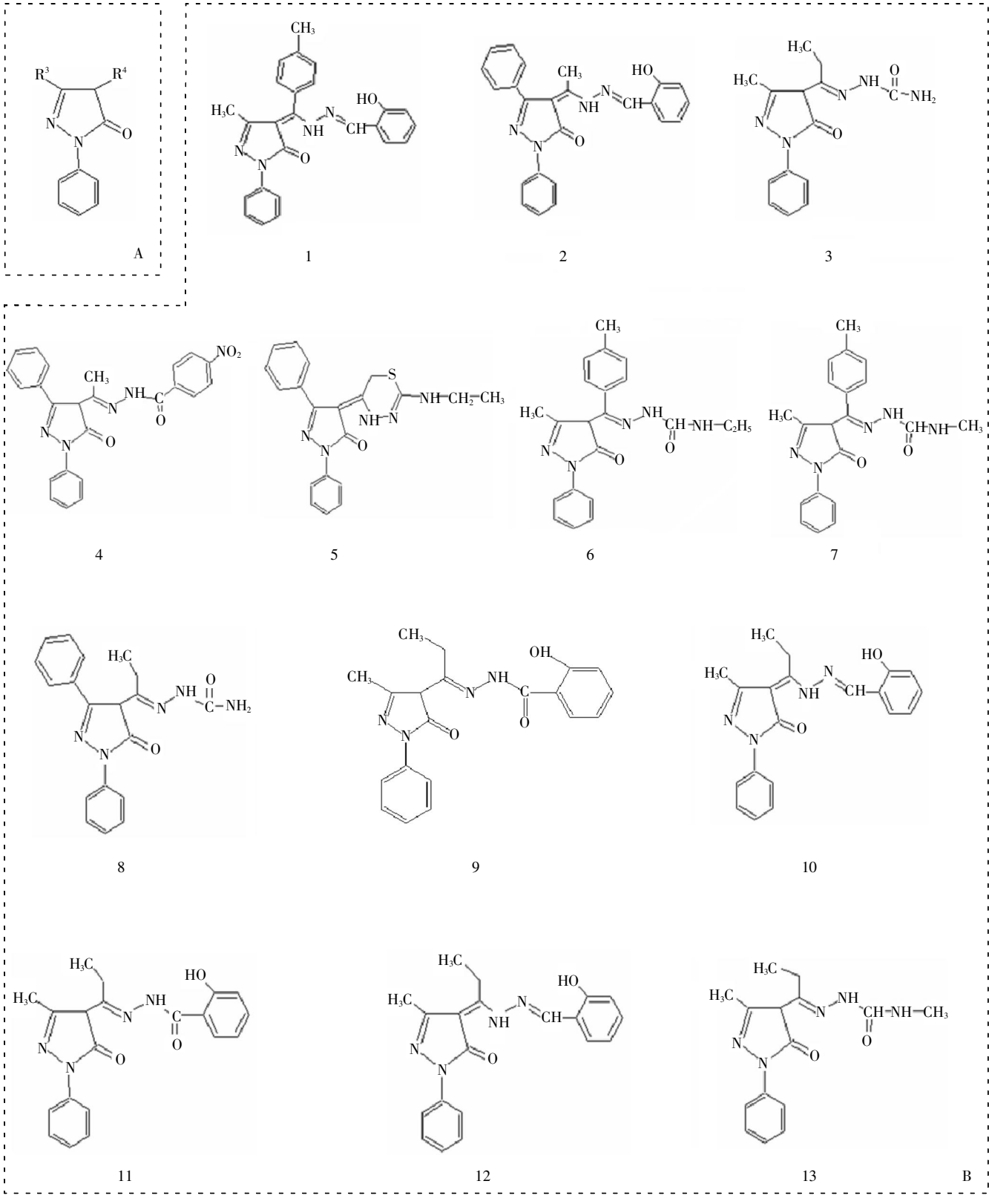

Figure 1 Chemical structure of the pyrazolone derivatives

A, basic chemical structure of the compounds; B, 1–13 are the numbers of compounds 1–13, respectively. Compounds 1–13 are 13 novel pyrazolone derivatives. Compounds 9 and 10 were copper (II) complexes with the same chemical structure with compounds 11 and 12 respectively.

tumor volume and treatment time. The mice were ethically killed when the mean tumor weight in the control group was over 1 g. Tumors were excised from the mice and their weight was measured. The rate of inhibition ratio (IR) was calculated according to the formula: IR = (1 – Mean tumor weight of the experimental group/Mean tumor weight of the control group) × 100%[12].

Cell viability assay

MTT assay measures the activity of mitochondrial dehydrogenase enzymes that cleave tetrazolium ring to produce formazan, thus the assay can be used as an index of cell viability. Briefly, cells were harvested during logarithmic growth phase and seeded into 96-well plates at a density of 1.5×10^4 cells/mL in a final volume of 190 μL per well. After 24 h of incubation, 10 μL of compounds full range concentration was added to 96-well plates. After 68 h of treatment, 10 μL MTT (stock solution 10 mg/mL of saline) was added to each well and the cells were incubated for 4 h at 37℃. Supernatant was removed and formazan crystals in viable cells were solubilized with 100 μL anhydrous DMSO. Thereafter, cell viability was measured by Model 550 Microplate reader at 540 nm with 655 nm as reference filter. All experiments were performed in triplicate. The 50% inhibitory concentration (IC_{50}) was determined as the anticancer drug concentration causing 50% reduction in cell viability and calculated from the cytotoxicity curves (Bliss's software)[13]. Cell survival was calculated using the following formula: survival rate = (mean experimental absorbance/ mean control absorbance) × 100%.

Statistical analysis

All experiments were repeated at least three times and statistical analysis was performed by *t*-test or one-way ANOVA with SPSS 11.0 software (SPSS Inc, USA). Signi ficance was determined at $P < 0.05$.

Results

Compound 9 exerted potent cytotoxicity against the four types of tumor cells in vitro

In this study, we investigated the cytotoxicity of 13 novel pyrazolone derivatives against four human tumor cells by MTT assay, including HepG2, OVCAR3, KB and multidrug resistance (MDR) KBv200 cells *in vitro*. Our experimental results showed that, of the 13 compounds, compounds 2, 5, and 13 showed no cytotoxic activity against the four types of cells (IC_{50} >100 μg/mL). Compounds 7 and 12 had poor activity against HepG2 and KBv200 cells respectively and no activity against others. Compounds 1 and 3 showed slight cytotoxicity against three other types of cells except HepG2 cells and compounds 6 and 8 showed slight cytotoxicity against three other types of cells except KB cells. Compounds 4 and 11 exhibited moderate activity against the four types of cells (Table 2).

Fortunately, we found that compound 10 exhibited significant cytotoxicity against the four types of cells, especially against HepG2 and OVCAR3 cells. We also found that compound 9 had the strongest effectiveness, of which IC_{50} values were (3.24 ± 0.28), (2.58 ± 0.61), (3.81 ± 0.02), and (3.45 ± 0.03) μg/mL in HepG2, OVCAR3, KB and MDR KBv200 cells, respectively (Table 2). It was important that the novel compounds 9 and 10 were able to overcome MDR, as well as P-gp type of resistance.

Cellular morphological changes induced by compound 9

To further observe the morphological characteristics of compound 9-induced cytotoxic effect in the four cell lines, cells were treated with the desired concentration of compound 9 (3–12 μg/mL) for 24 h and were observed by light microscopy. HepG2, OVCAR3, KB, and KBv200 cells exposed to compound 9 all displayed concentration-dependant damage. The control cells grew on the surface of the culture flask and a monolayer was formed. Cells treated with 3 and 6 μg/mL of compound 9 showed significant morphology changes that cells were detached from the culture surface and 12 μg/mL of compound 9 induced more severe cell damage. Notably, MDR KBv200 cells showed the similar morphology changes to those in KB cells exposed to the same concentration of compound 9 (Figure 2). The results suggested that compound 9 indeed had the marked cytotoxicity against the four types of human tumor cells and exerted the similar cytotoxic effect on both drug-sensitive KB cells and MDR KBv200 cells.

Compound 9 inhibited the proliferation of KBv200 cell xenografts in vivo

In the KBv200 cell xenograft experimental model, the tumor weight was (1.08 ± 0.54) g, (0.31 ± 0.27) g, (0.81 ± 0.44) g, (0.67 ± 0.31) g, and (0.57 ± 0.48) g in control group, CTX group, and 1.5 mg/kg, 3 mg/kg, and 6 mg/kg of compound 9 groups, respectively. The IR was 71.11%, 25.37%, 38.43% and 47.50% for CTX group and 1.5 mg/kg, 3 mg/kg, and 6 mg/kg of compound 9 groups, respectively (Table 3). The curves of tumor growth and the tumor size are shown in Figure 3.

In our previous study, we have demonstrated that compound 9 effectively inhibited tumor growth of KB cell xenografts in nude mice and indicated that the apoptotic molecular mechanism of compound 9 involved

Table 2 Cytotoxicity (IC_{50}) of 13 compounds against four types of human tumor cells

Compound	IC_{50} (μg/mL)			
	KB	KBv200	Hep-G2	OVCAR3
1	73.07 ± 20.33	50.86 ± 4.38	> 100	80.90 ± 13.76
2	>100	>100	> 100	> 100
3	96.18 ± 7.63	56.91 ± 2.61	> 100	66.66 ± 8.88
4	18.64 ± 4.71	48.68 ± 8.83	84.53 ± 6.72	60.74 ± 4.76
5	> 100	>100	> 100	> 100
6	> 100	69.79 ± 3.93	54.79 ± 6.68	81.58 ± 7.18
7	> 100	>100	89.73 ± 11.1	> 100
8	> 100	71.76 ± 5.32	31.53 ± 3.55	70.50 ± 1.84
9	3.81 ± 0.02	3.45 ± 0.03	3.24 ± 0.28	2.58 ± 0.61
10	41.73 ± 1.89	25.91 ± 1.22	4.41 ± 1.23	4.25 ± 0.40
11	35.19 ± 8.18	65.41 ± 9.48	59.52 ± 4.47	47.80 ± 5.25
12	> 100	61.03 ± 12.95	> 100	> 100
13	> 100	>100	> 100	> 100

Cells grew for 24 h and were exposed to a full range of concentrations of compounds 1–13 for 72 h. The cell density was assessed by Model 550 Microplate reader after stained with MTT for 4 h. Data are represented as mean ± standard deviation (SD) of at least triplicate determinations.

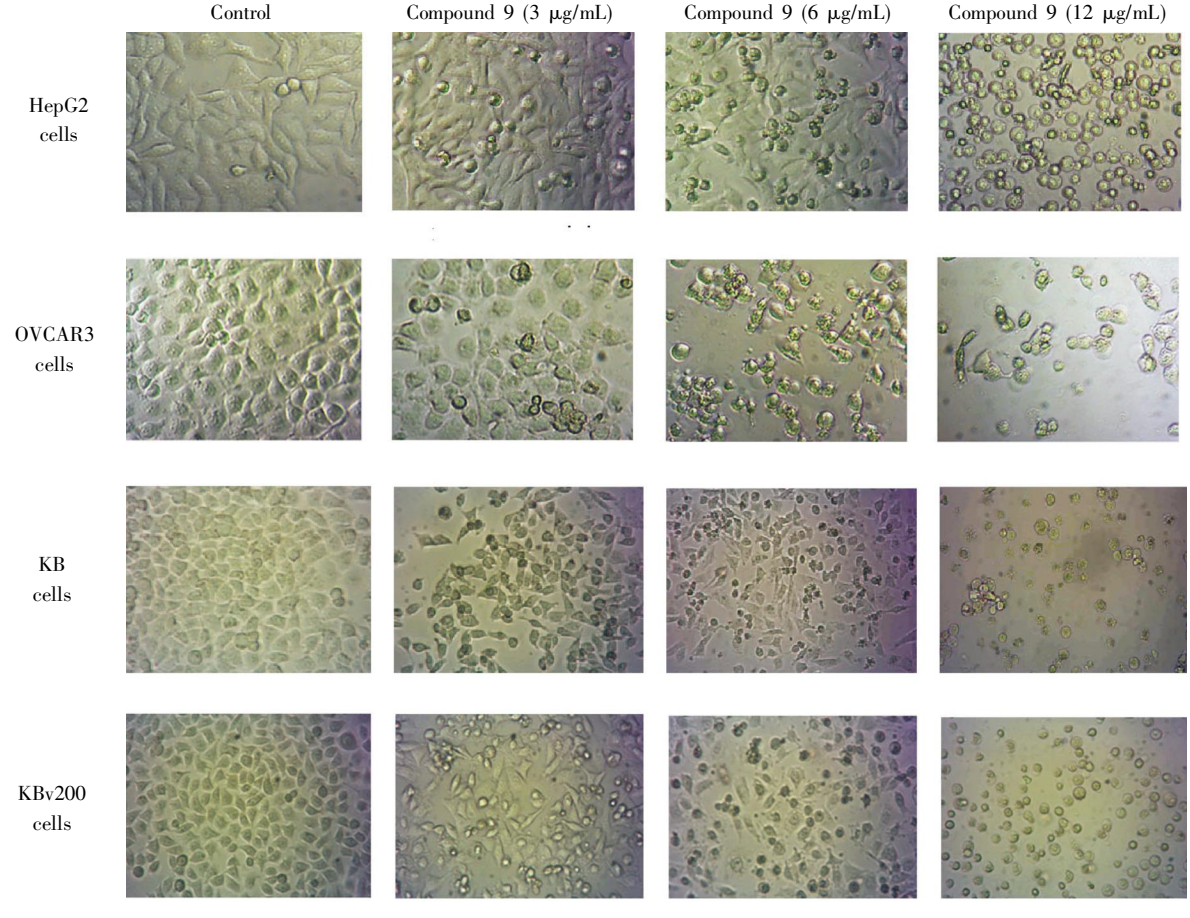

Figure 2 Damage effect of compound 9 on morphology of HepG2, OVCAR3, KB, and KBv200 cells

Cells were treated with 3–12 μg/mL of compound 9 for 24 h and were observed by light microscopy. Compared with the control groups, the groups treated with various concentrations of compound 9 displayed concentration-dependant morphology damage. Magnification of microscope was 100 folds.

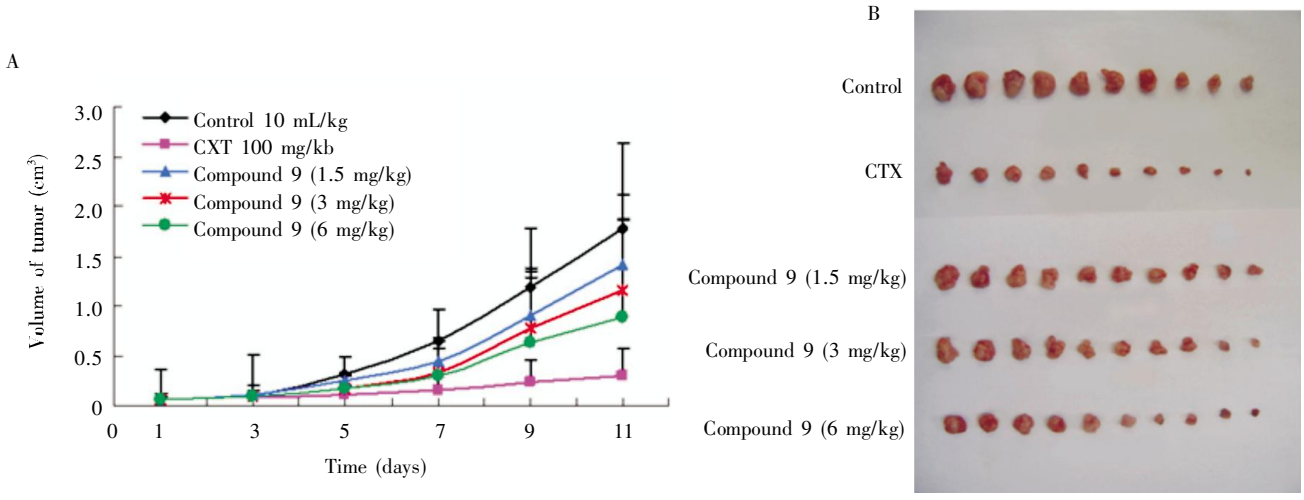

Figure 3 Inhibition of the growth of MDR KBv200 cell xenografts by compound 9 in nude mice

The experiment was carried out using athymic mice implanted subcutaneously (s.c.) with 2 × 10^6 cells under the right armpits. Animals were randomized into five groups including control (5% Tween 80 in saline), three desired concentrations of compound 9 (intraperitoneal (i.p.) injection, qd × 10) and CTX (i.p. injection, q5d × 2). Tumor growth was monitored starting on the first day of treatment and the volume of the xenografts was measured every 2 days. A, data are represented as means ± standard deviation (SD) of the tumor volume for each group of 10 experimental animals. B, the picture shows the tumor size at the end of the experiment.

Table 3 Inhibition of the growth of KBv200 cell xenografts by compound 9 in nude mice

Group	Pre-experiment		Post-experiment		Weight of tumor (g)	Inhibition ratio (%)
	n	Weight (g)	*n*	Weight (g)		
Control (10 mL/kg)	10	22.83 ± 2.28	10	24.74 ± 2.34	1.08 ± 0.54	
CTX (100 mg/kg)	10	21.96 ± 1.85	10	22.43 ± 2.13	0.31 ± 0.27[a]	71.11
Compound 9 (1.5 mg/kg)	10	21.47 ± 2.15	10	22.55 ± 2.1	0.81 ± 0.44	25.37
Compound 9 (3 mg/kg)	10	22.67 ± 2.79	10	23.31 ± 2.70	0.67 ± 0.31[b]	38.43
Compound 9 (6 mg/kg)	10	22.42 ± 2.33	10	22.67 ± 2.25	0.57 ± 0.48[a]	47.50

KB cells were implanted under the right armpits of athymic mice (subcutaneously injection). Animals were randomized into five groups including control (5% Tween 80 in saline), three desired concentrations of compound 9 (i.p. injection, qd × 10) and CTX (i.p. injection, q5d × 2). The weight of animals was measured every 2 days. Data are represented as mean ± SD.

[a] $P < 0.01$ and [b] $P < 0.05$ vs. the control by *t*-test or ANOVA.

ROS-independent mitochondrial dysfunction pathway. In the KB cell xenograft experiment, the IR was 77.42%, 22.98%, 34.68%, and 43.15% for CTX group and 1.5 mg/kg, 3 mg/kg, and 6 mg/kg of compound 9 groups, respectively [14]. The results indicated that compound 9 could effectively inhibit the xenografts proliferation not only in KB cells but also in MDR KBv200 cells. Importantly, the antiproliferative activity of compound 9 against MDR KBv200 cell xenografts was similar to that of compound 9 against drug-sensitive KB cell xenografts.

Discussion

The treatment of cancer with chemotherapeutic agents is frequently impaired or ineffective as a result of intrinsic or acquired MDR of the tumor cells. MDR is the phenomenon in which exposure of tumor cells to a single cytotoxic agent results in cross-resistance to other structurally unrelated classes of cytotoxics. And by far the most extensively characterized mechanisms is P-gp-mediated MDR. To develop new anticancer drugs which are efficient to MDR cells is a novel strategy to reverse the MDR phenotype.

Pyrazolone, as a prominent structural motif, is found in numerous active compounds. Due to the easy preparation and rich biological activity, pyrazolone framework plays an essential role and represents an interesting template for combinatorial and medicinal chemistry. Recent studies have suggested that several pyrazolone derivatives showed antiproliferative activity in several tumor cells [6,9,10]. In this study, we investigated the cytotoxicity of 13 novel pyrazolone derivatives against four human tumor cells by

MTT assay, including HepG2, OVCAR3, KB and multidrug resistance (MDR) KBv200 cells *in vitro*. Our experimental results showed that compound 9 (also named Lgf-YL-9[14]), a novel pyrazolone-based derivative, the IC_{50} values of which were (3.24 ± 0.28), (2.58 ± 0.61), (3.81 ± 0.02), and (3.45 ± 0.03) μg/mL in HepG2, OVCAR3, KB and MDR KBv200 cells, respectively, had significant cytotoxicity to the four types of tumor cells.

From the structure-activity relationship (SAR) study, we found that comparing with compound 4 (R^3 = phenyl; R^4 = (p-nitrobenzoylhydrazide)-ethidine), the activity of compound 2 (R^3 = phenyl; R^4 = (2- hydroxylbenzylidenehydrazine) -ethidine) was lost due to the different R^4. Introduction of R^4 in compound 1 (R^3 = methyl; R^4 = (2-hydroxylbenzylidenehydrazine)-p-methylbenzylidene) and compound 6 (R^3 = methyl; R^4 = (ethylaminoformylhydrazide) -p-methylbenzylidene) had more cytotoxic activity than did compound 7 (R^3 = methyl; R^4 = (methylaminoformylhydrazide) -p-methylbenzylidene).

As a result of different R^4, compound 9 (R^3 = methyl; R^4 = (salicyclidenehydrazide)-propylidene) demonstrated 7-fold more increase in cytotoxic activity in MDR KBv200 cells than did compound 10 (R^3 = methyl; R^4 = (2-hydroxylbenzylidenehydrazine)-propylidene). Strangely, in the case of compound 13 possessing (R^3 = methyl; R^4 = (methylaminoformylhydrazide)-propylidene), the cytotoxic activity was greatly less than that of compound 9, which indicated that R^4 of compound 13 resulted in an inactive agent and was also proved by R^4 of compound 7. It should be noted that compounds 9 and 10 had the same chemical structure with that of compounds 11 and 12 respectively, but they were copper (II) complexes which induced their more potent activity. As we know, in the light of the successful application of metal coordinated complexes in medicine, particularly as anticancer drugs, and in the search of more effective, less toxic, and wider spectrum chemotherapeutic agents, a group of copper-coordinated complexes have been developed[15]. Some links must exist in the properties of the metal ion and biological activity. It was reported that copper compounds exerted significantly antiproliferative activity and were more potent antitumor drugs, therefore further study for establishing their possible mechanism of action is warranted[16]. Zhang *et al.*[17] showed that *in vitro* tests, the copper (II) complexes exhibited cytotoxicity against murine leukemia P-388 and human leukemia HL-60 cell lines, which was more potent than the cytotoxicity cisplatin exhibited.

In summary, of 13 novel pyrazolone derivatives screened, compound 9 had the strongest cytotoxicity in this study. SAR studies indicated that the structure of R^4 and copper (II) seemed to be crucial for the cytotoxicity of compound 9. Furthermore, compound 9 showed the similar antitumor activity against drug-sensitive KB cells and MDR KBv200 cells *in vitro* and *in vivo*. So, compound 9 may be a promising antitumor agent benefiting cancer chemotherapy and provide an insight into the design of a novel leading compound for the development of MDR-overcoming antitumor agents.

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