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#### Synthesis, in vitro and in vivo anticancer activities of novel 4-substituted

#### 1,2-bis(4-chlorophenyl)-pyrazolidine-3,5-dione derivatives

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**Abstract:** To develop potent and selective anticancer agents, a series of novel 4-substituted 1,2-bis (4-chlorophenyl)-pyrazolidine-3,5-dione derivatives were designed and synthesized. All the compounds were evaluated for their antiproliferative activities against a panel of four human cancer cell lines. Among them, compound **4u** is the most potent, exhibiting  $IC_{50}$  values ranging from 5.1 to 10.1  $\mu$ M, respectively. Flow cytometry analysis and western blot analysis revealed that treatment of compound **4u** inducing MGC-803 cells cellular early apoptosis via activation of caspases-9/3. Furthermore, compound **4u** effectively reduced the tumor growth bared by human gastric cancer cells *in vivo* without obvious adverse side effects. Our findings indicate that compound **4u** may serve as a leading compound to target solid tumors.

**Keywords:** Pyrazolidine-3,5-dione; Antitumor; In vitro; In vivo; Caspases-9/3; Apoptosis

Cancer is a worldwide life-threatening disease, the cancer patients and the death cases are continually increasing in recent years, but the development of anticancer drugs with high efficiency and minimal side-effect remain to be a challenge.<sup>1</sup> Pyrazolidine

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diketones are a class of important nitrogenous heterocyclic compounds, which exhibit diverse biological activities in living organisms.<sup>2-4</sup> The biological properties of modified pyrazolidine diketones depend upon structural features of the heterocyclic ring system and side chains, providing a way for chemical modifications.<sup>5, 6</sup>

Since the phenylbutazone, a nonsteroidal anti-inflammatory agent had been synthetized in 1946, a series of improved agents have been discovered, such as oxyphenbutazone,  $\gamma$ -ketophenylbutazone, sulfinpyrazone (Figure 1). Moreover, pyrazolidine-3,5-dione derivatives have generated considerable interest lately due to their diverse biological activities, including the effect of anti-cardiovascular diseases, antihyperglycemic,<sup>5</sup> anti-tumour,<sup>7</sup> anti-HIV,<sup>3</sup> anti-inflammatory. Recently, pyrazoline-3,5-dione derivatives against COX-2 protein as well as marked inhibition of tumor progression and metastasis<sup>8-12</sup> have been reported, based on a close relationship between inflammation and cancer.<sup>13</sup>

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Regarding to the pharmacological importance of pyrazoline-3,5-dione functional group and continuation of our previous work<sup>14</sup> in developing novel anticancer derivatives on the side chains, we designed a series of 4-substituted 1,2-bis(4-chlorophenyl)-pyrazolidine-3,5-diones derivatives by an efficient synthetic, using different kinds of substituents  $R_1$  and  $R_2$ , and investigated their cytotoxic activities *in vitro* and *in vivo*. We found that compound **4u** significantly inhibited tumor growth *in vitro* and *in vivo* via inducing cell apoptosis.





#### compound.

The preparations of 4-substituted-1,2-bis (4-chlorophenyl)-pyrazolidine-3,5-diones were outlined in Scheme 1. Dimerization of *p*-chloroaniline, followed by Zn-mediated reduction gave hydrazine **2.** Condensation of **2** with diethyl malonate in the presence of MeONa afforded compound **3**. Condensation of compound **3** with aldehyde or ketone gave compounds **4a-w**. It is worth noting that no catalyst was needed for the condensation between **3** and aldehyde or ketone. All the synthesized compounds were fully characterized by <sup>1</sup>H NMR, <sup>13</sup>C NMR and high resolution mass spectra.



Scheme 1. Synthesis of compounds 4a-w. Reagents and conditions: (a)  $MnO_2$ , toluene, reflux; (b) Zn, NH<sub>4</sub>Cl, acetone, H<sub>2</sub>O, r.t; (c) NaOMe, diethylmalonate, EtOH, 50-140°C (dryness); (d) CH<sub>3</sub>OH, reflux.

All synthesized compounds were evaluated for their cytotoxic activities against four human cancer cell lines, including MGC-803 (gastric cancer), EC-109 (esophageal cancer), MCF-7 (breast cancer) and SMMC-7721 (hepatic cancer) and compared with the positive control 5-Fu (5-fluorouracil) by MTT assay<sup>15, 16</sup>. The IC<sub>50</sub> values of the tested compounds were listed in Table 1. As shown in Table 1, the cytotoxic activity of the target compounds against four human cancer cell lines with the IC<sub>50</sub> values ranging from 5.1 to 80.2  $\mu$ M. Among them, the most potent compound was **4u**, exhibiting IC<sub>50</sub> values ranging from 5.1 to 10.1  $\mu$ M, respectively.

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During the structure-activity relationship (SAR) analysis, we found that aromatic ring on the side chain of pyrazolidine-3,5-dione was important for antiproliferative acitivity: the aromatic ring derivative compounds **4s**, **4t**, **4v** and **4w** were more potent than the alicyclic hydrocarbon derivative compounds **4a** and **4b**.

Compound	R <sub>1</sub>	R <sub>2</sub>	$IC_{50}(\mu M)^a$			
			MGC-803	EC-109	MCF-7	SMMC-7721
4a	CH <sub>3</sub>	CH <sub>3</sub>	39.0 ± 1.1	42.1 ± 1.6	39.6 ± 1.1	$45.6 \pm 1.2$
4b	$\mathrm{CH}_3$	CH <sub>2</sub> CH <sub>3</sub>	$66.5~\pm~1.3$	41.1 ± 1.6	$41.6~\pm~1.8$	$49.2~\pm~1.7$
4c	Н	4-OH-C <sub>6</sub> H <sub>4</sub>	$19.5~\pm~0.9$	$34.2~\pm~1.1$	$23.2~\pm~0.8$	$20.6~\pm~1.2$
4d	Н	$4-N(CH_3)_2-C_6H_4$	$80.2~\pm~1.4$	> 128	> 128	> 128
4e	Н	4-F-C <sub>6</sub> H <sub>4</sub>	$26.0~\pm~1.0$	$35.6~\pm~0.6$	$31.4~\pm~1.1$	$23.3~\pm~1.2$
4f	Н	$4-Cl-C_6H_4$	$32.6~\pm~1.2$	$17.3~\pm~1.2$	$28.5~\pm~0.9$	$30.9~\pm~0.9$
4g	Н	$4\text{-Br-}C_6\text{H}_4$	$16.9 \pm 1.4$	$12.3~\pm~0.7$	$30.4~\pm~1.1$	$16.6 \pm 1.3$
4h	Н	3,4,5-(OCH <sub>3</sub> ) <sub>3</sub> -C <sub>6</sub> H <sub>2</sub>	$17.3~\pm~1.4$	$10.1~\pm~0.7$	$16.2~\pm~1.3$	$29.5~\pm~1.1$
4i	Н	3-OCH <sub>3</sub> -4-OH-C <sub>6</sub> H <sub>3</sub>	$21.8~\pm~1.1$	$7.1~\pm~0.5$	$22.8~\pm~1.0$	$64.9~\pm~1.4$
4j	Н	3-OCH <sub>3</sub> -2-OH-C <sub>6</sub> H <sub>3</sub>	$24.3~\pm~1.2$	39.0±1.1	$26.7~\pm~1.0$	$64.9~\pm~1.4$
4k	Н	$4\text{-OCH}_3\text{-}C_6\text{H}_4$	> 128	> 128	> 128	> 128
41	Н	3-Cl-C <sub>6</sub> H <sub>4</sub>	$14.4~\pm~1.2$	$22.6~\pm~0.7$	$18.3~\pm~1.0$	$20.2~\pm~1.0$
4m	Н	3-OCH <sub>3</sub> -C <sub>6</sub> H <sub>4</sub>	$26.6~\pm~1.0$	$29.0~\pm~1.2$	$18.1~\pm~1.5$	$30.2~\pm~1.3$
4n	$\mathrm{CH}_3$	$4-OH-C_6H_4$	$42.8~\pm~1.2$	$55.6~\pm~1.3$	$76.5~\pm~1.1$	$57.0~\pm~1.4$
40	Н	3,4-(OCH <sub>3</sub> ) <sub>2</sub> -C <sub>6</sub> H <sub>3</sub>	> 128	> 128	> 128	> 128
4p	Н	2,3-(OCH <sub>3</sub> ) <sub>2</sub> -C <sub>6</sub> H <sub>3</sub>	$29.6~\pm~0.9$	$6.8~\pm~0.2$	$11.9~\pm~0.7$	$14.1~\pm~0.8$
4q	Н	$2\text{-OCH}_3\text{-}C_6\text{H}_4$	$28.6~\pm~1.3$	$30.4~\pm~1.5$	$26.3~\pm~1.3$	$25.8~\pm~1.0$
4r	Н	2,4-(Cl) <sub>2</sub> -C <sub>6</sub> H <sub>3</sub>	> 128	$60.2~\pm~1.3$	> 128	> 128
<b>4s</b>	Н	3,4-(F) <sub>2</sub> -C <sub>6</sub> H <sub>3</sub>	$35.7~\pm~1.2$	$13.1~\pm~0.7$	$22.0~\pm~0.9$	$35.8~\pm~1.2$
4t	Н	2-furanyl	$12.8~\pm~1.1$	$14.3~\pm~0.5$	$20.3~\pm~1.0$	$19.5~\pm~1.0$
4u	Н	styryl	$5.1 \pm 0.7$	$9.9~\pm~0.4$	$9.4~\pm~0.6$	$10.1~\pm~0.2$
4v	Н	3-indolyl	$21.8~\pm~1.1$	$23.4~\pm~1.0$	$34.2~\pm~0.9$	$19.6~\pm~1.2$
4w	Н	C <sub>6</sub> H <sub>5</sub>	$27.2~\pm~1.1$	$32.7~\pm~0.7$	$20.2~\pm~1.2$	$26.2~\pm~1.1$
5-Fu			$9.2 \pm 0.7$	$15.9 \pm 0.9$	$13.5 \pm 1.0$	$14.2~\pm~1.3$

Table 1. Antitumor activity of compounds 4a-w.

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<sup>a</sup> Inhibitory activity was assayed by exposure for 72 h to substances. Data are presented as the means  $\pm$  SDs of three independent experiments.

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To evaluate the importance of the electronegativity and position of substituent groups on the aromatic ring for the antiproliferative acitivity, compounds **4f**, **4g**, **4l**, and **4w** were synthesized. Introduction of a weak electron-withdrawing group, a *para*-chlorine atom (**4f**), decreased the activity relative to **4w** on three cancer cell lines, except EC-109 cells. Moving the chlorine from the *para*-position (**4f**) to the *meta*-position (**4l**) enhanced cytotoxicity activity. In addition, adding halogen size from chlorine (**4f**) to bromine (**4g**) increased cytotoxicity activity on three cancer cell lines, except MCF-7 cells.

The importance of the substitution pattern and the number of methoxy groups on the phenyl ring of our compounds was also explored. The results showed that trimethoxy substitution on the 3'4' and 5'-positions of the phenyl ring (**4h**) was well tolerated. And comparison to the unsubstituted benzene derivative **4w**, the trimethoxy substitution increased cytotoxicity activity substantially. Further, moving methoxy group from the *para*-position (**4k**) to the *ortho*-position (**4m** and **4p**) produced an increase of the antiproliferative activity on the four tested cell lines.

The cytotoxicity of compound 4u against human normal gastric epithelial cell (GES-1) and the cell viability, using different concentrations of compound 4u, was also evaluated. As demonstrated in Figure 2, the cell viability decreased significantly with the increasing concentrations of compound 4u in cancer cells. However, in the normal gastric epithelial cell, the viability dropped slowly. The viability of human normal gastric epithelial cell stood at around 75% even treated with 20  $\mu$ M of 4u, indicating the selectivity of compound 4u against the tested cancer cells.



Figure 2. Selectivity of 4u on the cancer cell lines and human normal gastric epithelial cell line. (A) Cell viability was measured by MTT assay. (B) Value of  $IC_{50}$  was summarized with histogram graphs. The results were calculated by Graphpad prism software. Data are means  $\pm$  SDs of three independent experiments.

Apoptosis is considered a major way of most of the anticancer drugs. On the basis of the strong cytotoxicity of compound **4u** in MGC-803, then compound **4u** was chosen to further explore its mechanism of action. After treatment with **4u** at the indicated concentrations for 12 h, morphology changes of MGC-803 cells were recorded using an inverted microscope (Figure 3A). Besides, nuclear morphological changes at the corresponding concentration were also recorded and qualitatively evaluated by means of Hoechst 33258 fluorescent staining (Figure 3B) after incubation for 12 h at the indicated concentrations. Typical apoptotic markers, including cell rounding, nuclear condensation, nuclear fragmentation and apoptotic bodies were detected, especially at the highest concentrations of **4u**.



**Figure 3.** MGC-803 cells treated by **4u** showed typical apoptotic morphologies. (A) Morphology changes in MGC-803 cells treated with **4u** at the indicated concentrations. (magnification 200×). (B) Effects of **4u** on the nuclear morphology of MGC-803 cells were assayed by Hoechst 33258 staining. (magnification 200×).

In order to better characterize the mode of cell death induced by compound 4u, Annexin V-FITC/PI double staining was also applied, and a flow cytometer was used for quantitive analysis for apoptotic cells. As shown in Figure 4, when the cells were exposed to compound 4u for 12 h and 24 h, early apoptosis rates were extended from 6.3% (control) to 30.1%, and from 6.0% (control) to 52.7%, respectively. The results indicate that compound 4u markedly increased the cellular apoptosis in a concentration and time dependent manner.



**Figure 4.** Apoptosis effect on human MGC-803 cell line induced by compound **4u**. (A) Incubated for 12 h; (B) Incubated for 24 h. (C and D) Percentages of cells with apoptosis were summarized with histogram graphs. \*\*P <0.01 was considered statistically highly significant.

To further illustrate the mechanism of compound  $4\mathbf{u}$  induced apoptosis, we examined the effect of  $4\mathbf{u}$  on the activation of Caspase-9/3. Figure 5 showed that  $4\mathbf{u}$ significantly increased the expression of cleaved Caspases-9/3 and decreased the expression of their proenzymes. Meanwhile, the key proteins in the mitochondria-related apoptotic pathway were investigated. We found that the proapoptotic protein, Bax, was upregulated and the anti-apoptotic protein, Bcl-2, was downregulated in a concentration dependent manner after 24 h treatment of compound  $4\mathbf{u}$  (Figure 6). Our findings indicate that compound  $4\mathbf{u}$  may be involved in the mitochondria-related apoptosis.

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**Figure 5.** Expression of Cleaved-caspases-9/3 and Pro-caspase-9/3 in MGC-803 cells after treatment by compound **4u** for 24h. (A) Expression level of Cleaved-caspases-9/3 and Pro-caspase-9/3 were determined by western blot. (B) Densitometry quantitation of Cleaved-caspases-9 with indicated treatment. (C) Densitometry quantitation of Cleaved-caspases-3 with indicated treatment. Total levels of GAPDH were used as loading control. \*P <0.05 was considered statistically significant. \*\*P <0.01 was considered statistically highly significant.



Figure 6. Expression of Bax and Bcl-2 in MGC-803 cells after treatment by compound 4u for 24h. (A) Expression level of Bax and Bcl-2 were determined by western blot. (B) Densitometry quantitation of Bax with indicated treatment. (C) Densitometry quantitation of Bcl-2 with indicated treatment. Total levels of GAPDH were used as loading control. \*P <0.05 was considered statistically significant. \*\*P <0.01 was considered statistically highly significant.

In vivo inhibitory effect of compound **4u** on tumor growth was examined in a xenograft model. Xenograft tumors were generated by subcutaneous implantation of MGC-803 cells into nude mice. After the treatment of compound **4u** by oral administration, the mouse body weights were monitored and the tumor sizes were measured and recorded every 4 days (Figure 7A). After 28 days of treatment, compound **4u** significantly delayed the growth of tumor and reduced average tumor weight by 60.4%, which is very close to the effect of the positive control capecitabine (reduced average tumor weight by 62.7%) at the dose of 30mg/kg/d (Figure 7A, B and D). And there was no apparent body weight loss during the treatment (Figure 7C). These data indicate that compound **4u** was efficacious in inhibiting the growth of gastric tumor in vivo, but no obvious global toxicity.



Figure 7. In vivo antitumor effects of compound 4u in MGC-803 bearing nude mice. (A) Tumor volume with the indicated treatment. (B) Represented tumors with the indicated treatment. (C) Body weight with the indicated treatment. (D) Tumor weight with the indicated treatment. \*P <0.05 was considered statistically significant. \*\*P <0.01 was considered statistically highly significant. Data are mean  $\pm$  SDs.

In conclusion, twenty-three novel 4-alkylidene(arylidene)-1,2-bis(4-chlorophenyl)pyrazolidine-3,5-dione derivatives were designed and synthesized from commercially available *p*-chloroaniline. All synthesized compounds were evaluated for their antitumor activities against four human cancer cell lines. Among them, the most potent compound was **4u**, exhibiting  $IC_{50}$  values ranging from 5.1 to 10.1  $\mu$ M, respectively. Further investigation showed that compound **4u** caused the cellular apoptosis via the activation of capspase-9/3 in a time and concentration dependent manner. Meanwhile, compound **4u** showed efficacious activity in inhibiting the growth of gastric tumor *in vivo* with no obvious global toxicity. Synthesis of more analogs, SAR studies and further mechanism investigations are under way and will be reported in due course.

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### **References:**

1. S. H. Chan, C. H. Chui, S. W. Chan, S. H. L. Kok, D. Chan, M. Y. T. Tsoi, P. H. M. Leung, A. K. Y. Lam, A. S. C. Chan, K. H. Lam and J. C. O. Tang, *ACS Medicinal Chemistry Letters*, 2013, **4**, 170-174.

2. Nobuyoshi Takahashi, Hirofumi Nakagawa, Yoshinori Endo, US: 6180568, 1999, 01, 30.

3. B. A. Ten, D. W. Dekkers, S. M. Notten, M. L. Karsten, E. R. de Groot and L. A. Aarden, *Eur Cytokine Netw*, 2005, **16**, 144-151.

4. G. Deng, W. Li, J. Shen, H. Jiang, K. Chen and H. Liu, *Bioorg Med Chem Lett*, 2008, **18**, 5497-5502.

5. A. M. Gilbert, A. Failli, J. Shumsky, Y. Yang, A. Severin, G. Singh, W. Hu, D. Keeney, P. J. Petersen and A. H. Katz, *J Med Chem*, 2006, **49**, 6027-6036.

6. K. A. Koo, N. D. Kim, Y. S. Chon, M. Jung, B. Lee, J. H. Kim and W. Song, *Bioorg Med Chem Lett*, 2009, **19**, 2324-2328.

7. G. J. Kapadia, M. A. Azuine, Y. Shigeta, N. Suzuki and H. Tokuda, *Bioorg Med Chem Lett*, 2010, **20**, 2546-2548.

8. Q. J. Cao, M. H. Einstein, P. S. Anderson, C. D. Runowicz, R. Balan, J. G. Jones, *Int J Gynecol Pathol*, 2002, **21**, 147-154.

9. C. Denkert, M. Kobel, S. Berger, A. Siegert, A. Leclere, U. Trefzer, S. Hauptman, *Cancer Res*, 2001, **61**, 303-308.

10. R. N. DuBois, A. Radhika, B. S. Reddy, A. J. Entingh, *Gastroenterology*, 1996, 110, 1259-1262.

11. K. Uefuji, T. Ichikura and H. Mochizuki, Clin Cancer Res, 2000, 6, 135.

12. D. J. A. de Groot, E. G. E. de Vries, H. J. M. Groen and S. de Jong, *Critical Reviews in Oncology/Hematology*, 2007, **61**, 52-69.

13. M. Tsujii, S. Kawano and R. N. DuBois, Proc Natl Acad Sci U S A, 1997, 94, 3336-3340.

14. Q. R. Zhang, Y. Ke, Y. F. Cao, H. M. Liu, C. F. Wang, W. Y. Cheng, and M. L. Liu, *J Zhengzhou university (Med Sci)* 2010, **45**, 147-149 (in Chinese).

15. D. Horowitz and A. G. King, J Immunol Methods, 2000, 244, 49-58.

16. K. P. Putnam, D. W. Bombick and D. J. Doolittle, Toxicol In Vitro, 2002, 16, 599-607.

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