Asian Journal of Chemistry; Vol. 25, No. 9 (2013), 4885-4888

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.14135

Synthesis and Antitumor Evaluation of One Novel Tetramethylpyrazine-Rhein Derivative

PENG-LONG WANG, YA-TAO CHENG, KUO XU, YA-WEN AN, WEI WANG, QU-SHENG LI, QIU-JUN HAN, QIANG LI, HONG-GUI ZHANG and HAI-MIN LEI*

School of Chinese Pharmacy, Beijing University of Chinese Medicine, Beijing 100102, P.R. China

*Corresponding author: Tel: +86 10 84738641; E-mail: hm_lei@yahoo.cn

(*Received*: 29 May 2012; *Accepted*: 8 March 2013) AJC-13095

To discover multi-effective and low toxic anticancer lead compound from traditional Chinese medicine prescription, we synthesized one novel tetramethylpyrazine-rhein derivative which was composed of two main antitumor ingredients of Huazhenghuisheng Pian. The design idea was enlightened by "combination principle" in drug discovery and "compatibility principle" in traditional Chinese medicine. Compound **5**'s antitumor activity was evaluated by human cancer cell Bel-7402, while the angiogenesis activity was valued by the chick chorioallantoic membrane (CAM) assays. The stability test was carried on artificial gastric juice and artificial intestinal juice; furthermore, compound **5**'s acute toxicity was evaluated *in vivo*. The results showed that compound **5** not only displayed antiproliferative activity on Bel-7402 cell (IC_{50} = 26.4 μM), but also dramatically suppressed normal angiogenesis in chick chorioallantoic membrane. And there was no hydrolysis or structural damage during 24 h in artificial digestive solution. The LD_{50} value of the compound **5** exceeded 3.2 g/kg by oral administration in mice.

Key Words: Angiogenesis, Antitumor, Combination principles, Tetramethylpyrazine-rhein derivative, Multi-effective, Low toxicity, TCM.

INTRODUCTION

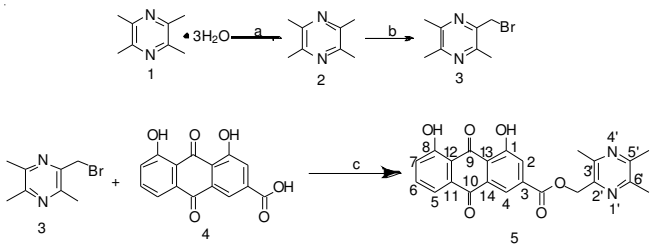
Due to following "compatibility principle" in traditional Chinese medicine (TCM) prescription, TCM uses multi-target effects to potentiate synergism of multi-effective compounds[1-3]. As its positive therapeutic effects, low toxicity and minimal side effects, TCM is increasingly accepted in the world[4-6]. At the application of structure combination idea, people recently exploited a novel field of lead compound discovery from TCMs. Zhang *et al.*[7,8] chose the major bioactive components in one classic TCM preparation to synthesize five novel esters connecting verticinone with bile acids, which showed satisfactorily effects and could be used as antitussive and expectorant agents in the future. We had chosen several effective antitumor ingredients from one TCM recipe to synthesize a series of ligustrazine derivatives. The target compounds exhibited more efficient, low toxic and multi-effective antitumor activities[9].

'Huazhenhuisheng Pian' is a classic traditional Chinese medicine recipe included in 'Pharmacopleia of the People's Republic of China' (2010 Edition), widely used to treat cancer in clinical[10-12]. Ligustrazine (TMP) and rhein (Rhe) were the main antitumor ingredients from this recipe[13-15]. Ligustrazine could not only be rapidly absorbed into blood but also pass through blood-brain barrier and blood-labyrinth barrier[16]. If coupled with Ligustrazine, the target compound could be convenient for administration and permeable to the physical barriers. As a part of our combination-idea on classic TCM preparation and to discover lead compound, we selected antitumor ingredients as pre-materials to synthesize TMP-Rhe derivative. The target compound's antitumor activity was evaluated on human cancer cell line of Bel-7402 by MTT assay. Its angiogenesis activity on the chick chorioallantoic membrane (CAM) assay was also evaluated.

EXPERIMENTAL

Reactions were monitored by TLC using silica gel coated aluminum sheets (Qingdao Haiyang Chemical Co., Qingdao, China) and visualized in UV light (254 nm). ^{1}H and ^{13}C NMR assays were recorded on a BRUKER AVANCE 500 NMR spectrometer (Fällanden, Switzerland) and chemical shifts are reported in δ (ppm). Mass spectra were obtained by using Q-TOF and (ESI^{+}) with an LC Autosampler Device: Waters 2695 instrument (New York, NY, USA). Melting points (uncorrected) were measured on an X-5 micro melting point apparatus (Beijing, China). Flash chromatography was performed using 300 mesh silica gel. The yields were calculated based on the last step reaction, synthesis route of compound **5** was shown in **Scheme-I**.

2-(Bromomethyl)-3,5,6-trimethylpyrazine (compound 3): Compound **1** (TMP·$3H_2O$, 5.000 g, 0.026 mol) was



Scheme-I: Synthesis route of compound **5**. Conditions and regents: (a) Benzene, reflux, 10 h, 100 %; (b) CCl_4, NBS, hv, reflux, 10-12 h, 70 %; (c) DMF, K_2CO_3, 85 ºC, 4 h, 31.4 %

dissolved in benzene (17 mL). The mixture refluxed for 10 h to evaporate the water of crystallization and compound **2** was gained. Compound **3** was prepared from compound **2** (TMP, 4.000 g, 0.029 mol) and N-bromosuccinimide (4.201 g, 0.024 mol) in refluxing carbon tetrachloride. The reaction mixture was illuminated by a 60W tungsten light bulb for 12 h. Then the mixture was filtered and the filtrate evaporated under vacuum and the crude oil-product was gained. Compound **3**, with 70 % purity, was not made further purification as it caused a strong mucous membrane irritation.

9,10-Dioxo-4,5-dihydroxy-9,10-dioxo-2-anthracenecarboxylic acid-3,5,6-trimethylpyrazin-2-methyl ester (compound 5): Compound **3** (2.320 g, 10.791 mmol) and Rhe (3.064 g, 10.791 mmol) were dissolved in dry DMF, then K_2CO_3 (3.475 g, 25.195 mmol) was added, the mixture was kept at 85 ºC for 4 h. The warm reaction mixture was poured into ice-water and the crude product was extracted with ethyl acetate. After drying the organic layer over anhydrous Na_2SO_4 and evaporating the solvent under vacuum, the crude product was separated by flash chromatography with petroleum ether:ethyl acetate (5:1) as eluent and recrystallized from acetone. Compound **5**: yellow crystals, m.p. 220.7-221.6 ºC, yield 31.4 %. ^{1}H NMR ($CDCl_3$) δ (ppm): 12.042 (s, 1H, 8-OH), 11.975(s, 1H, 1-OH), 8.434(s, 1H, 4-H), 7.878 (d, 1H, 5-H), 7.352 (d, 1H, 7-H), 7.746 (t, H, 6-H), 5.521 (s, 2H, O-CH_2), 5.52 (s, 2H, CH_2-2'), 2.64 (s, 3H, CH_3-6'), 2.59 (s, 3H, CH_3-5'), 2.56 (s, 3H, CH_3-3'). ^{13}C NMR ($CDCl_3$) δ (ppm): 162.4 (C1), 120.4 (C2), 137.8 (C3), 124.9 (C4), 120.4 (C5), 137.5 (C6), 125.5 (C7), 162.9 (C8), 192.8 (C9), 180.9 (C10), 134.0 (C11), 115.8 (C12), 118.4 (C13), 133.5 (C14), 164.1 (C15). Pyrazine ring: 66.5 (2'-CH_2), 151.7 (C2'), 144.3 (C3'), 149.0 (C5'), 149.5 (C6'), 21.6 (6'-CH_3), 21.5 (5'-CH_3), 20.5 (3'-CH_3). HRMS (ESI) m/z: 419.3173 $[M + H]^+$, calcd. (%) for $C_{23}H_{19}N_2O_6$ 419.1243.

Bio-evaluation methods

Cytotoxicity evaluation: The cytotoxicity of **5** was tested on human cancer cell line of Bel-7402 by the standard MTT assay. The cancer cell Bel-7402 was provided by Chinese Academy of Medical Sciences & Peking Union Medical College. The growing tumor cells at a density 10^4 cells/mL were exposed to various concentrations of the tested drugs and incubated in a 96-well microtiter plate for 96 h (37 ºC, 5 % CO_2). After MTT solution (20 µL, 5 mg/mL) was added to each well, the plate was incubated for a further 4 h. Then the media was removed. Formazan crystals were dissolved with DMSO (150 µL). After mixing well, the absorbance was quantified at 570 nm with a BIORAD 550 spectrophotometer. Wells containing no drugs were used as blanks. The IC_{50} values were defined as the concentration of compounds that produced a 50 % reduction of surviving cells and calculated using Logit-method. Tumor cell growth inhibitory rate was calculated in the following eqn. 1:

$$\text{Inhibition}\,(\%) = \left(1 - \frac{\text{Sample group OD}}{\text{Control group OD)}}\right) \times 100\ \%$$

Angiogenesis assay: Fertilized White Leghorn chicken eggs, provided by Chinese Academy of Agricultural Sciences, were placed in an incubator as soon as embryogenesis started and were kept under constant humidity of 65 % at 37 ºC. On day 7, a square window was opened on the shell and physiological saline (0.1 mL) was injected in to detach the shell membrane. Then gelatin sponges carrying the compound **5** stimulators at 10 and 40 µg/egg were implanted, respectively. The control group was treated with physiological saline. The windows were sealed with medical adhesive tape and the incubation went on till the experiment day. The above steps were performed under sterile condition. On the 11th day, the tapes were removed and the entire CAM was detached after tissue fixation with methanol/acetone (1:1, v/v). Then we used computer-assisted tracking of images to obtain absolute values for the number of microvessels which were 1-100 µm in diameter. Data were analyzed using *t*-test of statistics analysis system, the values were expressed as mean ± s of 6 observations and $p < 0.05$ was considered significant.

Stability test: According to "Pharmacopeia of the People's Republic of China" (2010 Edition), we prepared artificial gastric juice and artificial intestinal juice by dissolving pepsin and trypsase in pH = 1.2 and pH = 6.8 buffer, respectively. Then two samples of compound **5** (1.60 and 1.68 mg) were precisely weighed and mixed with 50 mL artificial gastric juice and 50 mL artificial intestinal juice, respectively. We keep the two artificial digestive solution in swing bed at 37 ºC and sampled 1 mL preparation at hour 0, 1, 2, 4, 6, 8, 12 and 24 h, respectively. Each sample was treated by the addition of 3 mL acetonitrile as protein precipitation agent. After centrifugation at 4500 rpm for 10 min, the supernatant was filtrated with 0.22 µm filtration film and then 10 µL filtrate was injected to HPLC analysis at a UV wavelength of 430 nm.

Acute toxicity: Kunming mice (Beijing Vital River Laboratory Animal Technology Company Limited, China) of both sexes, weighing 18-22 g, were divided into four groups of 10 animals matched in weight and size. The mice were placed in cages and kept under standard environmental conditions with a standard rodent diet and water *ad libitum* under a 12 h light-dark cycle. They were deprived of food for 24 h but allowed free access to tap water throughout the experiments. This research was carried out in accordance with the "Regulation for the Administration of Affairs Concerning Experimental Animals" (State Council of China, 1988).

The maximum suspended dose (80 mg/mL) of **5** was prepared in 0.3 % Na-CMC water solution, then one group of 20 both sexes mice were administered the maximum tolerated dose (0.4 mL/10 g) by oral administration. The other 20 mice, control group, were gave 0.3 % Na-CMC (0.4 mL/10 g) *via* gavage. The general behaviour of the mice was observed

continuously for 1 h after the treatment and then intermittently for 4 h and thereafter over a period of 24 h. The mice were further observed for up to 14 days following treatment for any signs of toxicity and deaths and the latency of death. Behavioural, toxic effects and mortality response were recorded.

RESULTS AND DISCUSSION

Compound **3** was synthesized by TMP and N-bromosuccinimide (NBS) *via* free radical reaction. The typical subsequent synthetic procedure involved the combination of bromo TMP and Rhe through ester-link under alkaline condition. Compound **3** and Rhe were dissolved in dry dimethyl formamide (DMF) at 85 ºC for 4 h with N_2 protection to give compound **5**. The target compound was elucidated by HRMS and NMR spectrometers. Because of the formation of hydrogen bonds with the neighbouring carbonyl group (CO), the two signals at δ 12.04 and δ 11.96 in the ^{1}H NMR correspond to the H-1 and H-8 protons. δ 192.8 (C(9)) and 180.9 (C(10)) in ^{13}C NMR are characteristic peaks of carbonyl groups (CO) in anthraquinone struture. Furthermore, HRMS (ESI) gave the molecular ion peak corresponding to the molecular weight of the confirmed compound **5**.

Biological activities

MTT test: The antiproliferation effects of **5** and reference materials were evaluated in Bel-7402 tumor cell using the MTT assay. As shown in Table-1, after combination with TMP, compound **5** (IC_{50} 26.4 μM) exhibited much better antitumor activities than compared materials.

TABLE-1
ANTIPROLIFERATIVE EFFECT OF COMPOUND **5** AND COMPARED MATERIALS

IC_{50} (μM)	Group			
	TMP	Rhe	5	CU[a]
Bel7402	48.8	34.6	26.4	18.5

[a]Worth mentioning is that curcumin (CU), which has been recognized as a potential chemoprevetative and chemotherapeutic agent[17], is the standard in Bel-7402 tumor cell test.

In addition, we found compound **5** revealed in a dose-dependent manner to Bel7402 cells (Fig. 1). It showed an inhibitory rate of 74.13 % when it was 50.0 μM. For 24 and 48 h, the inhibitory effects were similar to different concentrations of the compound **5**.

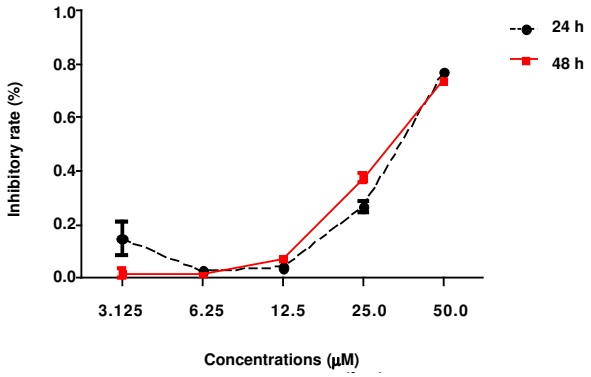


Fig. 1. Antiproliferative effect of **5** during 24 h and 48 h

Though compound **5**'s antitumor activity was a little lower than CU, CU was poorly available following oral administration to patients[18]. While both TMP and Rhe were available following oral administration[16,19]. Accordingly, compound **5** might have better oral absorbility than that of CU. Moreover, TMP could be rapidly absorbed to pass through blood-brain barrier and blood-labyrinth barrier[16], we expected compound **5** could also pass through blood-brain barrier and explore novel antitumor agent to cure brain cancer.

Angiogenesis activity: Antiangiogenesis as a way of treating primary tumors and reducing their metastases had been proposed by Folkman[20]. Clinical practice also proved that antiangiogenic drugs could enhance the treatment efficacy of cytotoxic chemotherapy[21]. Especially the multi-effective antitumor agents presented positive significance to cancerous persons. This was supported by compound Linifanib, which was a novel, orally active multi-targeted agent. Linifanib exhibited potent antitumor and antiangiogenic activities against a broad spectrum of experimental tumors and malignancies in patients[22]. According to the references, TMP could inhibit angiogenesis[23]. Consequently, compound **5**'s angiogenesis activity was evaluated by the chick chorioallantoic membrane (CAM) assay.

The model was established according to our previous work[9]. As shown in Fig. 2 and Table-2, this study directly showed that compound **5** could inhibit the angiogenesis of the CAM.

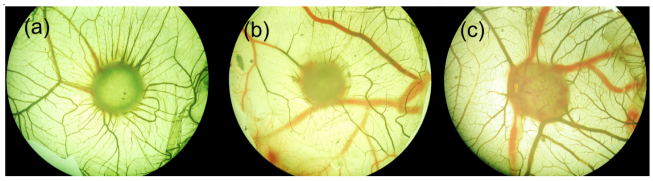


Fig. 2. Microvascular proliferation of compound **5** on CAM (× 50). (a) Control for compound **5** group, (b) 10 μg/egg for compound **5** group, (c) 40 μg/egg for compound **5** group

TABLE-2
MICROVASCULAR PROLIFERATION OF 5 ON CAM

Compound	Control ($\bar{X} \pm S$)	Treatment ($\bar{X} \pm S$)	Dose (μg/egg)	Egg (n)
5	9.0 ± 2.68	5.2 ± 1.60[a,*]	10	6
5	9.0 ± 2.68	5.8 ± 2.31[b]	40	6

[a,*]$p = 0.0299 < 0.05$, [b]$p = 0.0803 > 0.05$.

Stability test: Compound **5** was connected tetramethylpyrazine and rhein by ester bone and ester bone might be hydrolyzed by digestive enzyme following oral administration. So we evaluated compound **5**'s stability in 37 ºC artificial gastric juice and artificial intestinal liquid. As shown in Fig. 3, there was no hydrolysis or structural damage during 24 h in artificial digestive solution.

Acute toxicity: Oral therapeutic remedies are easy and convenient for the cancer sufferers[24]. Luckily, both tetramethylpyrazine and rhein are available following oral administration[16,19]. And compound **5** may have better oral absorbility. So we just evaluated the acute toxicity of compound **5** by gavage. During two weeks, there were no deaths or signs of toxicity observed after oral administrated maximum tolerated

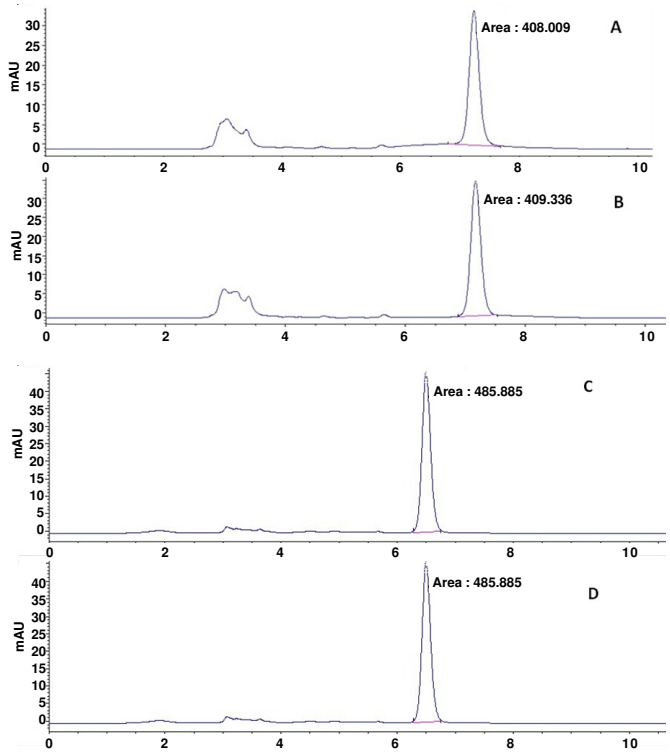


Fig. 3. HPLC chromatogram of compound **5** from artificial digestive solution. (A) 0 h of compound **5** in artificial gastric juice, (B) 24 h of compound **5** in artificial gastric juice, (C) 0 h of compound **5** in artificial intestinal juice, (D) 24 h of compound **5** in artificial intestinal juice

dose (3.2 g/kg). This proved that compound **5** have a low toxicity *in vivo*.

Conclusion

Tetramethylpyrazine-rhein derivative (**5**) was synthesized through conjugation of two main antitumor bioactive compounds from one classic TCM receipt. The final results showed that compound **5** not only inhibited proliferation of Bel7402 cancer cell but also dramatically suppressed new angiogenesis in CAM. In addition, the preliminary stability test displayed that compound **5** was stable in 37 ºC artificial gastric juice and artificial intestinal liquid during 24 h. Furthermore, the acute toxicity assay of compound **5** indicated no toxicity. Studies on pharmacology and acute toxicity of compound **5** give us new hints for anticancer drug discovery and development.

The above design idea is enlightened by "combination principle" in drug discovery and "compatibility principle" in TCM prescription. Both of the two principles are aim to unite the different pharmacodynamic components to be a whole and to maximize the pharmacological effects. "Huazhenhuisheng Pian", a complex traditional Chinese medicine formulation, is widely used to treat cancer in Chinese communities. But this TCM formulation confronts many daunting challenges, such as the indistinct basal pharmacodynamic materials, standardization problems due to the natural variability of the crude materials and the chemical complexity of the preparation. All of these had blocked it heading into the international medicinal market. However, compound **5**, one novel ester of tetramethylpyrazine and rhein, will make standardization and preparation problems simpler because it is a single compound. The results of the experiment suggest that the attempt to apply structure combination to discover multi-effective and low toxic antitumor lead compounds from traditional Chinese medicine formulations is viable.

ACKNOWLEDGEMENTS

This study was financially supported by the National Creating New Drug Program of China (2009ZX09103-356), National Natural Science Foundation of China (No. 81173519) and the Innovation Team Project Foundation of Beijing University of Chinese Medicine (Lead Compounds Discovering and Developing Innovation Team Project Foundation, the project number No. 2011-CXTD-15).

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