Synthesis and biological evaluation of 4-substituted aryl-piperazine derivatives of 1,7,8,9-tetrachloro-10,10-dimethoxy-4-azatricyclo [5.2.1.0^{2,6}] dec-8-ene-3,5-dione as anti-cancer and anti-angiogenesis agents

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Abstract

Background: A series of aryl-piperazine derivatives of 1,7,8,9-tetrachloro-10,10-dimethoxy-4-azatricyclo [5.2.1.0^{2,6}] dec-8-ene-3,5-dione were synthesized. The chemical structures of the desired compounds were identified by ¹H NMR, ESI-MS and elementary analytical. The anti-cancer and anti-angiogenesis activities of the newly synthesized compounds were evaluated by proliferation and migration assays, respectively. **Results:** The screening results demonstrated that compounds 2 and 5 showed potent anti-tumor activity (IC₅₀ values ranging from 7.1 to 15.9 μM) with low cytotoxic activities (IC₅₀ = 79.3 μM). Although compound 5 showed little effects on endothelial proliferation (IC₅₀ = 65.3 μM), it indeed significantly abrogated endothelial cell migration (IC₅₀ = 6.7 μM). **Conclusions:** This work may impart new direction for the investigations of aryl-piperazine derivatives and lead to the development of potent novel anti-tumor and anti-angiogenesis agents.

Background

The structure of polycyclic fused pyrrolidine ring system with a rigid bowl-shaped skeleton have attracted great interest and become as an important and challenging synthetic endeavor [1, 2]. As a result, X-azatricyclo [m.n.0.0^{a,b}] alkanes, namely fused pyrrolidine ring systems, are frequently encountered basic skeleton in many synthetically challenging and bioactive alkaloids [3-6]. Due to its facility of constructing two rings simultaneously, intramolecular 2,3-dipolar cycloaddition strategies have become an important tool for the synthesis of structurally complex fused heterocyclic ring systems [7]. The Diels-Alder reactions between N-substituted maleimide and cyclopentadiene compounds is a powerful method for the synthesis of fused pyrrolidine ring systems. We envisaged an azatricyclic structural entity, the construction of 4-azatricyclo (5,2,1,0^{2,6}) alkanes, via the intramolecular [3+2]-dipolar cycloaddition reaction of N-substituted maleimide with cyclopentadiene. In addition, Kossakowski group demonstrated that the rigid arylcyclo analogues having azatricyclo ring systems showed wide ranging pharmacological activities, such as anti-viral, anti-HIV, and antibacterial [8-12].

Nowadays, little research was directed towards 4-substituted aryl-piperazine compounds at fused pyrrolidine ring as a new therapeutic agent for the treatment of cancer and angiogenesis. Therefore, we synthesized a series of 1,7,8,9-Tetrachloro-10,10-dimethoxy-4-azatricyclo [5.2.1.0^{2,6}] dec-8-ene-3,5-dione derivatives and tested as a prominent anti-cancer agent in various types of human cancer cell lines. The best compounds 2 and 5 showed potent anti-cancer activity and were further characterized by both anti-proliferation and anti-migration assays.

Experimental Section

Materials and experimental conditions

All reagents and solvents were commercially available and either used without further purification or purified by standard methods before use. Melting points were measured using a melting point detector

(RY-1, Jinshi, China). ^1H NMR spectra were on a nuclear magnetic resonance spectrometer (JNM.ECA-400, JEOL, Japan) at 400MHz in the solvent indicated below. Mass spectra were obtained from high resolution magnetic mass spectrometer (ZabSpec, Micromass, UK) and mass spectrometer (API-3000, AB Sciex, USA). Elemental analysis was determined by the elemental analyzer (CarloErba1106, Rodano, Italy). The synthetic pathway for the title compound was outlined in **Scheme 1**.

Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Inc. (Kumamoto, Japan). Human lung cancer cell lines (A549), Human hepatoma cell lines (HepG2), human prostate cancer cell lines (PC3), human umbilical vein endothelial cells (HUVECs), and Human lung fibroblasts (HFL-1) were supplied by Cell Resource Center of Peking Union Medical College. Thalidomide and methotrexate (98%, purity) were acquired from Sigma Aldrich (St. Louis, MO, USA). To rule out the effect of DMSO on cell proliferation, the maximum concentration of DMSO was <0.03%. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, and penicillin streptomycin were purchased from Hyclone (Logan, UT, USA).

Synthesis and characterization

Synthesis

Both N-R-maleimide and 5,5-dimethoxy-1,2,3,4-tetrachlorocyclopentadiene were dissolved in anhydrous toluene (100 ml). Then, the solution was refluxed for 8 h. After the solvent was removed under reduced pressure, the residue was dissolved in ether (150ml), washed with water and brine, dried over anhydrous sodium sulfate, and concentrated to dryness. The product was purified by flash-chromatography (petroleum ether/ethyl acetate, 6:1) and the title compound was isolated.

Characterization of 1,7,8,9-tetrachloro-4-ethyl-10,10-dimethoxy-4-azatricyclo (5,2,1,02,6) dec-8-ene-3,5-dione (1)

White solid, yield 94%, mp: 118 ~ 120°C. ^1H -NMR (ppm, CDCl_3 , 25 °C) δ : 3.58(m, 2H), 3.39 (6H, s), 3.12 (d, 2H), 1.25 (t, 3H). ESI-MS: 507.5 (M+1). Anal. Calcd. (%) for $\text{C}_{13}\text{H}_{13}\text{Cl}_4\text{NO}_4$: C, 40.13; H, 3.37; N, 3.60. Found (%): C, 40.19; H, 3.40; N, 3.32.

Characterization of 1,7,8,9-tetrachloro-4-(3,5-Dichlorobenzyl)-10,10-dimethoxy-4-azatricyclo (5,2,1,02,6) dec-8-ene-3,5-dione (2)

White solid, yield 82%, mp: 104 ~ 106°C. ^1H -NMR (ppm, CDCl_3 , 25 °C) δ : 7.55 (m, 2H), 7.13 (s, 1H), 3.63 (s, 6H), 3.18(d, 2H). ESI-MS: 507.5 (M+1). Anal. Calcd. (%) for $\text{C}_{17}\text{H}_{11}\text{Cl}_6\text{NO}_4$: C, 40.35; H, 2.19; N, 2.77. Found (%): C, 40.42; H, 2.21; N, 2.73.

Characterization of 1,7,8,9-Tetrachloro-4-benzyl-10,10-dimethoxy-4-azatricyclo (5,2,1,02,6) dec-8-ene-3,5-dione (3) [14]

White solid, yield 76%, mp: 113 ~ 115 °C. ¹H-NMR (ppm, CDCl₃, 25 °C) δ: 7.37 (m, 2H), 7.27 (m, 3H), 4.55 (2H, s), 3.63 (s, 3H), 3.62 (s, 2H), 3.55 (s, 3H). ESI-MS: 452.6 (M+1). Anal. Calcd. (%) for C₁₈H₁₅Cl₄NO₄: C, 47.92; H, 3.35; N, 3.10. Found (%): C, 47.81; H, 3.41; N, 3.22.

Characterization of 1,7,8,9-Tetrachloro-4-phenyl-10,10-dimethoxy-4-azatricyclo (5,2,1,02,6) dec-8-ene-3,5-dione (4)

White solid, yield 81%, mp: 121 ~ 124 °C. ¹H-NMR (ppm, CDCl₃, 25 °C) δ: 7.43 (m, 3H), 7.13 (m, 2H), 3.80 (2H, s), 3.68 (s, 3H), 3.61 (s, 3H). ESI-MS: 458.9 (M+1). Anal. Calcd. (%) for C₁₈H₁₅Cl₄NO₄: C, 46.71; H, 3.00; N, 3.20. Found (%): C, 46.80; H, 3.11; N, 3.17.

Characterization of 1,7,8,9-tetrachloro-4-(2-fluorobenzyl)-10,10-dimethoxy-4-azatricyclo (5,2,1,02,6) dec-8-ene-3,5-dione (5) [15]

White solid, yield 94%, mp: 106 ~ 108 °C. ¹H-NMR (ppm, CDCl₃, 25 °C) δ: 7.81 (m, 1H), 7.17 (m, 1H), 7.01 (m, 1H), 6.99 (m, 1H), 3.35 (s, 6H), 3.12 (d, 2H). ESI-MS: 456.7 (M+1). Anal. Calcd. (%) for C₁₇H₁₂Cl₄FNO₄: C, 44.87; H, 2.66; N, 3.08. Found (%): C, 44.92; H, 2.69; N, 3.02.

Characterization of 1,7,8,9-tetrachloro-4-(p-methoxyphenyl)-10,10-dimethoxy-4-azatricyclo (5,2,1,02,6) dec-8-ene-3,5-dione (6)

White solid, yield 94%, mp: 126 ~ 128 °C. ¹H-NMR (ppm, CDCl₃, 25 °C) δ: 7.57 (m, 2H), 6.98 (m, 2H), 3.63 (s, 3H), 3.42 (s, 6H), 3.12 (d, 2H). ESI-MS: 468.3 (M+1). Anal. Calcd. (%) for C₁₈H₁₅Cl₄NO₅: C, 46.28; H, 3.24; N, 3.00. Found (%): C, 46.32; H, 3.29; N, 2.96.

Biological activities

Anti-cancer activity

HepG2, PC3, A549, HFL-1 cells were maintained in DMEM and HUVECs were HUVECs in F12K supplemented with 10% FBS and a dual antibiotic solution containing ampicillin (100 U/mL) and streptomycin (100 mg/mL) at 37°C in a humidified atmosphere under 5% CO₂. The cells in logarithmic growth phase were harvested for the following experiments. Compounds 1-6 were dissolved in DMSO at a concentration of 200.0 mM stock solution. The stock was diluted by PBS to the required concentration immediately before use.

CCK-8 assay

HepG2, PC3, A549 (2×10³ cells/well), HFL-1 (5×10³ cells/well), and HUVECs (9×10³ cells/well) cells were seeded in 96-well plate and cultured in the growth medium supplemented with 10% FBS. Cells were allowed to adhere and recover overnight, and then replaced with fresh culture medium (DMEM or F12K, 1% FBS). Cells were treated with PBS, indicated doses of methotrexate and compounds 1-6. After 72 h,

10 µl of CCK-8 solution was added to each well and incubated for 2 h at 37 °C. Then, the absorption was measured by the microplate at 450 nm (Bio-Rad Laboratories, Hercules, CA, USA).

Cell migration assay

HUVECs cells were measured by using a Transwell assay. Briefly, HUVECs cells were seeded into the upper chamber of Transwell filters at the final density of 1×10^5 (Costar, New York, NY, USA) with DMEM (FBS free). Lower chambers were filled with DMEM supplemented with 0.2% FBS containing Compounds 1-6 or thalidomide. After culturing for 6 hours, the cells migrated to the bottom of the membrane were fixed with 10% formalin and stained with 0.1% crystal violet. The number of migrated cells was obtained by counting in five random fields under a light microscope. Experiments were independently repeated three times.

Statistical analysis

The data were expressed as the mean $\pm$ standard deviation (SD). Basic statistical analyses were performed with the software SPSS version 23.0 (IBM, New York, NY, USA) and GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Both one-way ANOVA and Student's t-test were used to determine significance. A p-value less than 0.05 were considered statistically significant.

Results And Discussion

Synthesis

The synthetic route of the 1,7,8,9-tetrachloro-4-R-10,10-dimethoxy-4-azatricyclo (5,2,1,0^{2,6}) dec-8-ene-3,5-dione derivatives was outlined in *Scheme 1*. As reported, the synthesis of 1,7,8,9-tetrachloro-4-R-10,10-dimethoxy-4-azatricyclo (5,2,1,0^{2,6}) dec-8-ene-3,5-dione begins with the interaction of addition N-R-maleimide and addition 5,5-dimethoxy-1,2,3,4-tetrachlorocyclopentadiene with the help of heating reflux in anhydrous toluene to give the target compounds [13]. The compounds (1,2,4,6) are reported for the first time. All the synthesized compounds 1-6 (Table 1) gave satisfactory elementary analytical and spectroscopic data. ¹H NMR and ESI-MS spectra were consistent with the specified structures. The configuration of the cycloalkene skeleton of the imide R is *endo, cis* [10]. Among them, compounds 3 and 5 were further determined by an X-ray crystallography study [14, 15].

Biological activities

The introduction of a halogen group in a strategic position of a molecule seemed to be a very powerful and versatile tool for the exploitation of compounds with biological activities [16,17]. The presence of a halogen influences the properties of functional groups nearby, exerting the dipole moment of the molecule, changing its acidity or basicity, as well as reactivity [16]. The insertion of electron withdrawing fluorine and chlorine atoms into a drug modulates the steric and electronic parameters that leads to risen lipophilicity, thus improves rates of absorption and transport of drugs [17]. Consistently, our results

demonstrated that of the six compounds synthesized, compounds with halogen substitutions (2, 5) showed good antitumor and an endothelial cells migration activity.

Previously, several studies reported that the rigid arylcyclo analogues exerts its anti-tumor activity. To determine the anti-cancer viability of the synthesized compounds, CCK-8 assay was used to evaluate the cell viability in human hepatoma cell lines HepG2, PC3 and A549. The concentrations of the title compounds and methotrexate (positive control) with 50% inhibition (IC_{50}) on the cells were evaluated, and the results were summarized in Table 1. Among the synthesized compounds, both compounds 2 and 5 displayed the best anti-tumor activity with IC_{50} values ranging from 7.1 to 15.9 μ M. Then, we further evaluated the growth inhibitory activities of six compounds against the HFL-1 cell lines. As mentioned in Table 2, compounds 2 and 5 exhibited weak cytotoxicity with IC_{50} values of 79.3 and 88.5 μ M on HFL-1, respectively, which suggested compounds 2 and 5 might be an ideal anti-cancer candidate with weak toxicity on normal cells.

Recently, several studies demonstrated that anti-angiogenesis therapy was an effective way to abrogate tumor growth. We wondered whether both compounds 2 and 5 showed anti-angiogenetic activity? Anti-proliferation and anti-migration assays were applied. Both compounds 2 and 5 only showed marginally effect on the proliferation of HUVECs, and the IC_{50} values were 51.5 μ M and 65.3 μ M, respectively. However, compounds 2 and 5 significantly repress the migration of HUVECs, with the IC_{50} values were 9.5 μ M and 6.7 μ M, respectively (Table 3). The anti-migration effects of compound 5 was almost the same as that of the thalidomide group (Figure 1). Collectively, our data suggest that compound 5 could inhibit tumor growth not only by direct targeting tumor cell, but also abrogating migration of endothelial cells.

Conclusion

In summary, the synthesis of title compounds was produced by Diels-Alder reactions between N-substituted maleimide and cyclohexadiene compounds under mild reaction conditions. The anti-tumor and anti-angiogenesis properties of the six compounds were evaluated. Our results demonstrated that compounds 2 and 5 were either inactive or only weakly active against normal HFL-1 and HUVECs cell proliferation, whereas compounds 2 and 5 showed promising anti-tumor and anti-endothelial cells migration activities. These results provide an encouraging framework that could lead to the development of potent the anti-tumor and anti-angiogenesis agents.

Declarations

Authors' contributions

He Liu designed the study. Lifang Guo and Benshan Xu performed the most experimental works as well as wrote the manuscript draft. Lihong Liu covered all financial support for this project. Zirui Wan and Lulu Ren did the biological part and helped in its' writing. Jie Zhang and Wen Zhang helped in statistical analysis. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All the data and material support this manuscript are available either in the article or attached as additional file.

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Tables

Table 1. Structure of compounds

Entry	R—	Compd.	Structure
1	C ₂ H ₅ —	1,7,8,9-tetrachloro-4-ethyl-10,10-dimethoxy-4-azatricyclo (5,2,1,0 ^{2,6}) dec-8-ene-3,5-dione	
2		1,7,8,9-tetrachloro-4-(3,5-Dichlorobenzyl)-10,10-dimethoxy-4- azatricyclo (5,2,1,0 ^{2,6}) dec-8-ene-3,5-dione	
3		1,7,8,9-Tetrachloro-4-benzyl-10,10-dimethoxy-4-azatricyclo (5,2,1,0 ^{2,6}) dec-8-ene-3,5-dione	
4		1,7,8,9-Tetrachloro-4-phenyl -10,10-dimethoxy-4-azatricyclo (5,2,1,0 ^{2,6}) dec-8-ene-3,5-dione	
5		1,7,8,9-tetrachloro-4-(2-fluorobenzyl)-10,10-dimethoxy-4- azatricyclo (5,2,1,0 ^{2,6}) dec-8-ene-3,5-dione	
6		1,7,8,9-tetrachloro-4-(p-methoxyphenyl)-10,10-dimethoxy-4- azatricyclo (5,2,1,0 ^{2,6}) dec-8-ene-3,5-dione	

Table 2. Cell growth Inhibition (IC₅₀, μ M) of all target compounds against human cancer cell lines

Compd.	HepG2	PC3	A549
1	53.7	30.9	22.4
2	11.7	15.9	11.3
3	17.2	21.4	28.1
4	21.1	16.1	22.4
5	7.1	9.9	10.8
6	29.8	16.9	20.4
Methotrexate	7.5	4.3	9.5

Table 3. Cell growth Inhibition (IC₅₀, μ M) of all target compounds against HFL-1

Compd.	1	2	3	4	5	6	Methotrexate
HFL-1	100.1	79.3	50.3	89.9	88.5	71.9	19.3

Figures

Figure 1

Figure 1

Transwell migration assay using HUVECs cells. (A) Attractants include cell-free culture media (F12K/ antibiotics) (Mock); cell-free culture media plus 0.2% FBS (FBS (0.2%)); cell-free culture media plus 0.2% FBS and 5 μ M compound 2 (Compound 2); cell-free culture media plus 0.2% FBS and 5 μ M compound 5 (Compound 5); cell-free culture media plus 0.2% FBS and 5 μ M thalidomide (thalidomide); original magnification: 9200 (a). Data are presented as the number of cells that migrated to the bottom of an 8-mm pore membrane (b). Values are expressed as mean $\pm$ SD (**, $P < 0.01$; ***, $P < 0.005$).

Supplementary Files

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