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Synthesis and biological evaluation of quinoxaline-5,8-diones that inhibit vascular smooth muscle cell proliferation

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Abstract—A series of 6-arylamino-2,3-bis(pyridin-2-yl)-7-chloro-quinoxaline-5,8-diones were synthesized and evaluated for their inhibitory activity on rat aortic smooth muscle cell (RAoSMC) proliferation. The quinoxaline-5,8-diones exhibited a potent antiproliferative activity. Further mechanistic study revealed that the inhibitory effect of one representative quinoxaline-5,8-dione on SMC proliferation was mediated by modulation of the extracellular signal-regulated kinase 1/2 signaling pathway in the RAoSMCs.

1. Introduction

The abnormal proliferation and migration of vascular smooth muscle cells (SMCs) play a pivotal role in progression of coronary artery atherosclerosis and restenosis following angioplasty.[1] Arterial injury results in the migration of SMCs into the intimal layer of the arterial wall, where they proliferate and synthesize extracellular matrix components. Several growth factors induce the proliferation and migration of arterial SMCs.[2] Platelet-derived growth factor (PDGF) is one of the most potent promoters of the proliferation and migration of SMCs.[3]

Heterocyclic quinonoid compounds are an attractive class of biologically active molecules.[4] Therefore, we synthesized and tested various quinone derivatives to elucidate their contribution to the antiproliferative effects on PDGF-stimulated SMC proliferation. Among the quinones tested, quinoxaline-5,8-dione derivatives **1** (Fig. 1) showed potent antiproliferative activity.

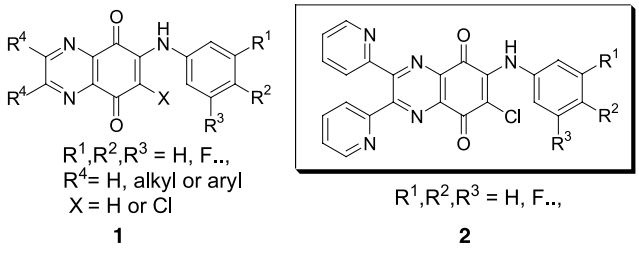

Figure 1. Quinoxaline-5,8-dione derivatives.

There have been several reports[5–10] on some quinoxaline-5,8-dione derivatives which exhibited antibacterial[5] and antimalarial[6] effects, cytotoxic activity[7] against cancer cell lines, and antiasthmatic and antiallergic activity.[8]

However, the antiproliferative activity on the SMCs of quinoxaline-5,8-diones has not been reported yet to the best of our knowledge. We describe herein our preliminary results on the synthesis of 6-arylamino-2,3-bis(pyridin-2-yl)-7-chloro-quinoxaline-5,8-diones **2** and their antiproliferative activity on the rat aortic SMCs (RAoSMCs).

Additional mechanistic study of SMC antiproliferative activity by compound **2c** was also performed. The mito-

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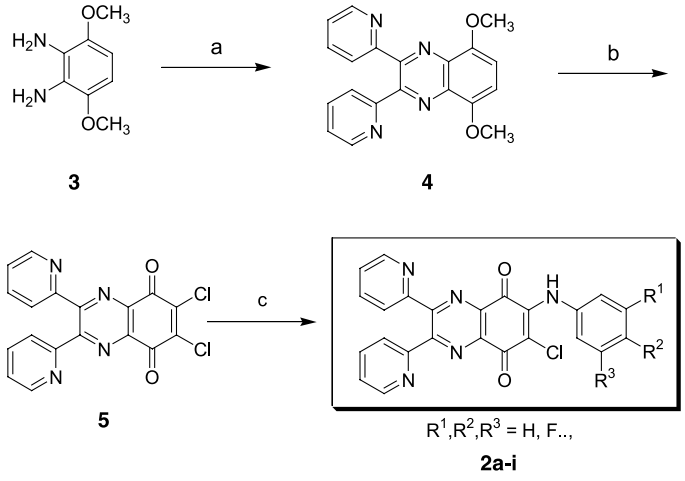
gen-activated protein kinase (MAPK) cascade known as the extracellular signal-regulated kinase (ERK) pathway mediates mitogenic responses induced by a wide variety of growth factor receptors in many cell types, including SMCs.[9] MAPKs play an important role in regulating cell growth and survival, and are also involved in both mitogenic and stress responses of cells.[10] MAPK is activated through a specific phosphorylation cascade. In general, the ERK pathway plays a major role in regulating cell growth and differentiation, being highly induced in response to growth factors and cytokines.[11] The ERK pathway is required for cell cycle arrest, apoptosis and growth of the SMCs.[12] Along this line, we determined whether the inhibitory effect of quinoxaline-5,8-diones on the proliferation of SMCs is related to the modulation of ERK activation and cell cycle arrest using a representative compound **2c**.

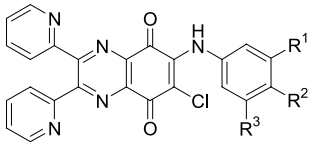
2. Chemistry

The method used to synthesize the 6-arylamino-2,3-bis(pyridin-2-yl)-7-chloro-quinoxaline-5,8-diones **2** is shown in Scheme 1. 2,3-Diamino-1,4-dimethoxybenzene (**3**) was prepared according to the known method with minor modifications.[13] Cyclizations of compound **3** with 2,2′-pyridil gave 2,3-bis(pyridin-2-yl)-5,8-dimethoxy-5,8-dihydroquinoxaline[14] (**4**), resulting in 91% yields. 2,3-Bis(pyridin-2-yl)-6,7-dichloro-quinoxaline-5,8-dione (**5**) was synthesized by oxidizing compound **4** with HNO_3/HCl combination in 89% yields. 6-Arylamino-2,3-bis(pyridin-2-yl)-7-chloro-quinoxaline-5,8-diones **2a**–**i** (Table 1) were prepared by nucleophilic substitution on compound **5** with appropriate arylamines. Most of these substitutions went as expected and had overall high yields of 74–94%.



Scheme 1. Synthesis of 6-arylamino-2,3-bis(pyridin-2-yl)-7-chloro-quinoxaline-5,8-diones. Reagents and conditions: (a) 2,2′-pyridil/H_2O/rt/0.5 h/91%; (b) concd HCl/concd HNO_3/reflux/0.5 h/89%; (c) arylamine (1 equiv)/EtOH/reflux/5 h/74–94%.

Table 1. Structures and IC_{50} values of 6-arylamino-2,3-bis(pyridin-2-yl)-7-chloro-quinoxaline-5,8-diones for inhibition of SMC proliferation



Compound	R^1	R^2	R^3	SMC[a] IC_{50}[b] (μM)
2a	H	Cl	H	1.5
2b	H	OH	H	5.5
2c	H	F	H	1.0
2d	H	CF_3	H	1.1
2e	H	OCF_3	H	1.0
2f	H	OCH_3	H	3.5
2g	H	H	H	3.1
2h	Cl	Cl	H	1.0
2i	F	F	F	1.2
4				>100
MPA				1.0

[a] The SMCs were isolated from rat thoracic aorta.
[b] The inhibitory activity against the PDGF-induced proliferation of the SMCs.

3. Biological activity

6-Arylamino-2,3-bis(pyridin-2-yl)-7-chloro-quinoxaline-5,8-diones **2** were evaluated in vitro for their antiproliferative activity on the RAoSMCs. Inhibition of proliferation of these cells was determined by WST colorimetric assay.[15,16] The IC_{50} values were determined and compared to the positive control mycophenolic acid[17] (MPA). As shown in Table 1, most of the test compounds exhibited good activity. In particular, quinoxaline-5,8-diones **2c**, **2e**, and **2h** revealed potent inhibitory activities and also were comparable to that of MPA. In contrast, compounds **2b**, **2f**, and **2g** exhibited relatively lower activity.

These results suggest that 6-arylamino-2,3-bis(pyridin-2-yl)-7-chloro-quinoxaline-5,8-diones **2** might be a promising lead for the development of potential inhibitors of SMC proliferations.

From the viewpoint of the structure–activity relationship there is no significant difference between the halide substituents (R: F, Cl, Br) in the 6-arylamino moieties of quinoxaline-5,8-diones **2**. In addition, the quinone moiety in quinoxaline-5,8-diones **2** might be essential for the antiproliferative activity, for example, as nonquinonoid compound **4** lost activity.

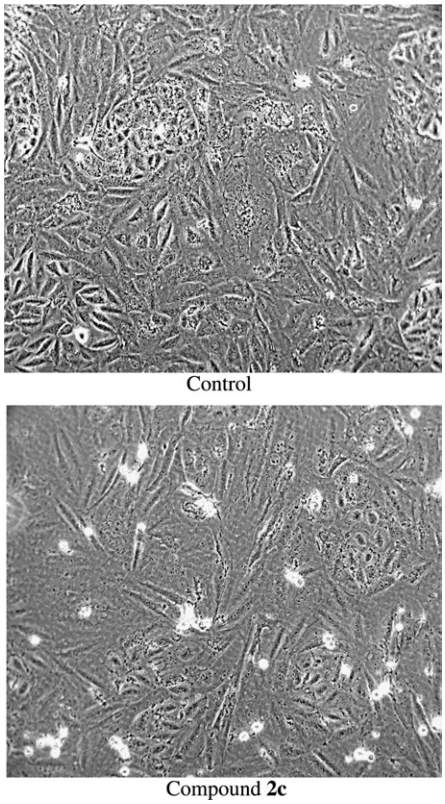


Figure 2. Morphological change in cultured SMCs treated with compound **2c**. SMCs treated with DMSO alone (control) or compound **2c** (1 μg/mL) for 48 h were observed under the phase-contrast microscope and photographed.

Further mechanistic study on the antiproliferative activity was performed using one of the potent compounds **2c** in cultured SMCs. As shown in Figure 2, when SMCs were treated with compound **2c** for 48 h morphological changes also revealed that the cell density was decreased by observation under the phase-contrast microscope.

To further explore whether the antiproliferative effects of compound **2c** were mediated by the modulation of the cell cycle in SMCs, DNA contents were analyzed by flow cytometry (Figs. 3A and B).[18] As illustrated in Figure 3, when SMCs were treated with compound **2c** for 48 h the DNA contents accumulated in the S phase of the cell cycle.

To investigate whether the cell cycle arrest by the test compound was related to the expression of cell cycle regulator proteins, Western blot analysis was performed.[19] As shown in Figure 4A, the level of cdk 2 was markedly reduced by the treatment of compound **2c**. In addition, the down-regulation of cyclin A, which binds to cdk 2 and promotes progression through the S

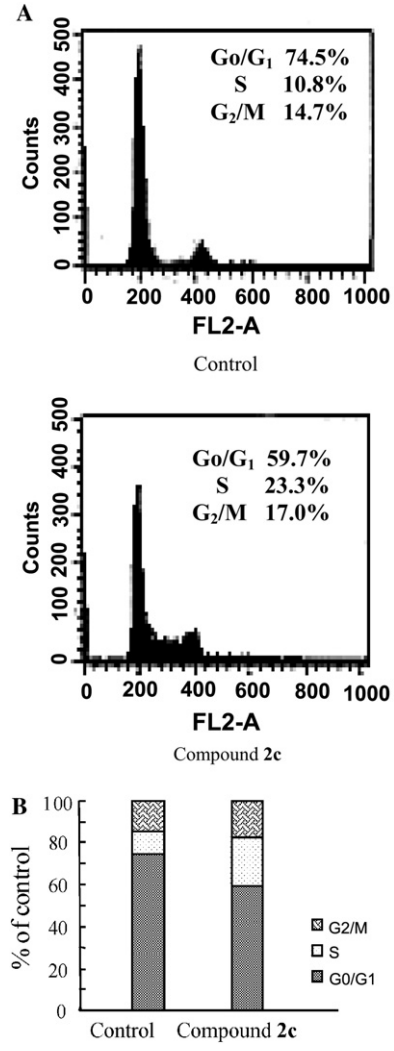


Figure 3. Effect of compound **2c** on cell cycle progression in cultured SMCs. Cells were treated with compound **2c** (1 μg/mL) for 48 h and then the cell cycle was analyzed by flow cytometry analysis.

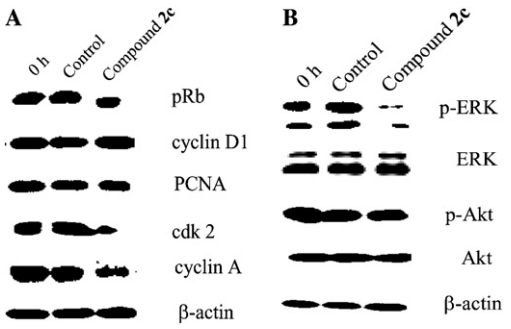


Figure 4. Effect of compound **2c** on protein expression in cultured SMCs. (A) Total cell lysates from SMCs treated with compound **2c** (1 μg/mL) for 48 h were analyzed for pRb, cyclin D1, PCNA, cdk 2, and cyclin A. (B) Compound **2c** was exposed to SMCs for 1 h, and the expression of phosphorylation of ERK and phosphorylation of Akt was examined.

phase of cell cycle, was also observed in compound **2c**-treated cells. However, the expression levels of pRb and cyclin D1, which are related to the progression of G1 into S phase were not much affected by the treatment of compound **2c**. These results indicate that the test compound might affect the exit of S phase cell cycle and thus accumulate the DNA contents of S phase in the cells.

In addition, to further explore the underlying mechanisms on the antiproliferative effect exerted by quinoxaline-5,8-diones we investigated ERK and Akt cell signaling pathways. As a result, a remarkable decrease of ERK (1/2) phosphorylation, but not Akt, was detected with the treatment of compound **2c** (1 μg/mL) for 1 h (Fig. 4B), indicating that the ERK signaling pathway might be involved in the inhibition of SMC proliferation by compound **2c**.

The ERK and Akt signaling pathways have been known to play pivotal roles in SMC proliferation. Several studies have indicated that the inhibition of SMC proliferation is ERK-dependent.[20] In this study, the regulation of ERK by the test compound **2c** was manifested, but not much related to the regulation of Akt. In this stage, the involvement of and relationship to ERK by Akt for the inhibition of SMC proliferation are unclear. Further study will be designed to clarify the relationship.

In conclusion, 6-arylamino-2,3-bis(pyridin-2-yl)-7-chloro-quinoxaline-5,8-diones are considered to be a new class of inhibitors of smooth muscle cell proliferation, and the antiproliferative effects might be associated with its blockade of cell cycle progression and suppression of ERK signaling activation in the cell. Further pharmacological investigations of these quinoxaline-5,8-diones and the structural optimization are in progress in our group.

4. Statistical analysis

The results are shown as means ± SD of the number (n) of experiments. Statistical analysis of difference was determined by a one-way analysis of variation (ANOVA). Differences were accepted as statistically significant at a p value of <0.05.

Acknowledgment

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15. *SMC proliferation assay*: The RAoSMCs were isolated from rat thoracic aorta by method described previously.[16] The cells were maintained in Dulbeccos's modified Eagle's medium (DMEM, Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/mL, and 100 μg/mL streptomycin. The SMCs were seeded in triplicate at a concentration of 1×10^3 cells/well in 200 μL of DMEM containing 10% (v/v) fetal bovine serum in 96-well flat-bottomed plates (Costar, Corning, NY, USA). After 24 h incubation, the complete medium was replaced with DMEM containing 0.2% FBS, and incubated for an additional 72 h. And then the cells were treated with test compounds in 100 μL of DMEM containing PDGF (5 ng/mL) and 5% (v/v) fetal bovine serum for 48 h. Proliferation of the cells was determined using a colorimetric assay kit based on the uptake of WST by viable cells (Premix WST-1 cell proliferation assay system, Takara Bio, Otsu, Japan). The assay kit is dependent on the reduction of tetrazolium salt WST-1, which results in formation of a dark red formazan product, by various mitochondrial dehydrogenase of viable cells.
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18. *Cell cycle analysis:* SMCs (1×10^6 cells) were seeded in 100-mm culture dish and incubated with 10% FBS DMEM. After 24 h, medium was changed in DMEM free, and SMC was further incubated for 24 h. Subsequently, SMC was again incubated in 10% FBS DMEM containing test sample **2c** (1 μg/mL). After 48 h of treatment, both floating and adherent cells were collected. The cell suspensions were then fixed with 70% ice-cold ethanol and transferred to the freezer until use followed by washing with PBS, cells were stained with 50 μg/mL propidium iodide (PI) in the presence of 0.25 mg/mL ribonuclease (RNase) at 37 °C for 30 min. Stained cells were analyzed by flow cytometry (Becton–Dickinson, USA).
19. *Western blot analysis:* SMC cultures were plated in 10 mm-culture dishes at a density of 1×10^6 cells/mL in DMEM containing 10% FBS. After 24 h incubation, the cells were washed twice with 5 mL PBS. The stimulation mixture contained vehicle only or the indicated concentration of test compound **2c** in supplemented DMEM and then incubated for 48 h. After washing with PBS twice, 1 mL boiling 2× concentrated electrophoresis sample buffer (1× = 125 mM Tris–HCl buffer, pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, and 1% β-mercaptoethanol) was added to the cells, which were then scraped from the dish and boiled for 5 min. Each protein (30 μg) was then subjected to SDS–polyacrylamide gel electrophoresis using 10% acrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, MA). After blocking, the membranes were incubated with antibodies (goat anti-pRb, mouse anti-PCNA, rabbit-anti cdk2, rabbit anti-cyclin A, and rabbit anti-cyclin B1, diluted 1:1000) or anti-β-actin (1:5000 dilution) for 1 h, and then incubated with a corresponding secondary antibody (anti-rabbi IgG-HRP, anti-mouse IgG-HRP, 1:2000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The blots were developed using an ECL detection kit (Amersham Life Science, Buckinghamshire, UK).
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