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Design, synthesis, and biological evaluation of *N*-arylpiperazine derivatives as interferon inducers

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Abstract

Type I Interferon (IFN) signaling plays an important role in the immune defense system against virus infection and in the innate immune response, thus IFNs are widely used as anti-viral agents and treatment for immune disorder or cancer. However, there is a growing demand for novel small-molecule IFN inducer due to tolerance, toxicity, or short duration of action following direct administration of IFNs. In this study, we assessed arylpiperazine (ARP) as a new core skeleton of IFN inducer. To investigate structure-activity relationship, we designed and synthesized a series of ARP analogues and evaluated the ability to stimulate IFN response in THP-1 human monocyte cells. Compound **5i** was identified as a potent type I IFN inducer as it significantly increased cytokine secretion and increased expression of various IFN-stimulating genes which are representative biomarkers of type I IFN pathway. Our results suggested a beneficial therapeutic potential of **5i** as an anti-viral agent.

Keyword: interferon inducer, type I Interferon, arylpiperazine, anti-viral agent, innate immunity

Highlight

- We designed and synthesized a series of arylpiperazine derivatives as interferon inducers
- We tested different linker system and carbonyl moiety resulted in increase of potency
- **5i** exhibited the activity of interferon inducer with EC_{50} of 13.1 μM and no cytotoxicity
- **5i** initiated immune responses by mediating type I IFN signaling

Interferons (IFNs) are essential signaling peptides which act as the first line of surveillant in defense system and modulate innate and adaptive immune activation[1,2]. In response to virus infection, IFNs are released by host cells, after which they bind to their specific receptors, thereby stimulating JAK/STAT signaling pathway and initiating transcription of various interferon-stimulated genes (ISGs) such as CXCL10, IRF7, IFIT3, and OAS etc [3–7]. Activation of IFN signaling pathway regulates not only anti-viral response but also induces cellular immunity by stimulating macrophage or monocyte [8].

Based on the class of binding receptor and signaling cascade, IFNs are categorized into three major group: type I, type II and type III. Type I IFNs including IFNα and IFNβ are considered as therapeutic targets for treatment of hepatitis B and C infections and multiple sclerosis [9,10]. In addition, IFNs are used in cancer treatments of hematological malignancy such as leukemia and lymphomas [11,12]. Despite their therapeutic potential, there are several limitations of IFNs treatments including toleration, dose-related toxicity and side-effects such as dizziness, headache, muscle pain, and depression [13]. Moreover, IFNs are typically administrated by intramuscular injection for therapeutic use. PEGylated IFNs with enhancing stability are generally used in clinical treatment to overcome the short duration of effect. However, PEGylated IFNs are not able to be used on patients with hyperbilirubinemia [14,15]. For these reasons, continuous efforts have been made to develop small-molecule modulators so as to stimulate IFNs. For example, tilorone is the first synthetic small-molecule drugs used as orally active IFN inducer which showed efficient anti-viral activity against broad spectrum of viruses including ebola virus and middle east respiratory syndrome-related coronavirus (MERS-Cov), especially [16].

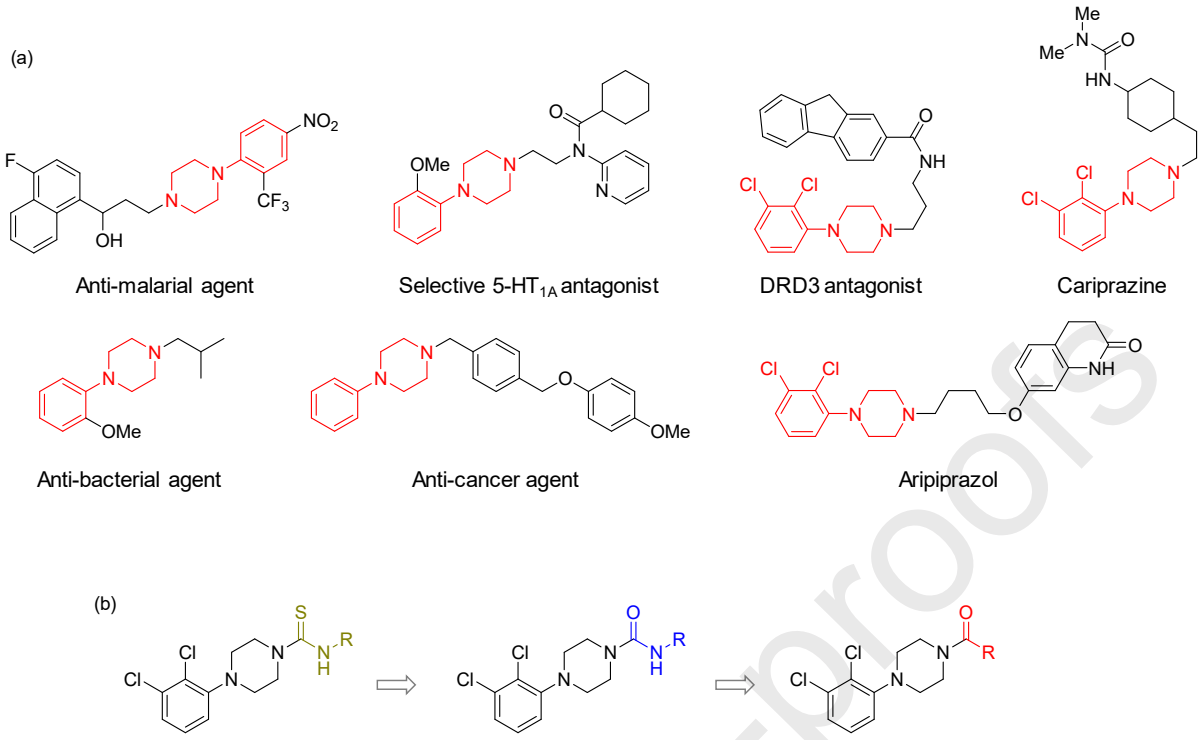
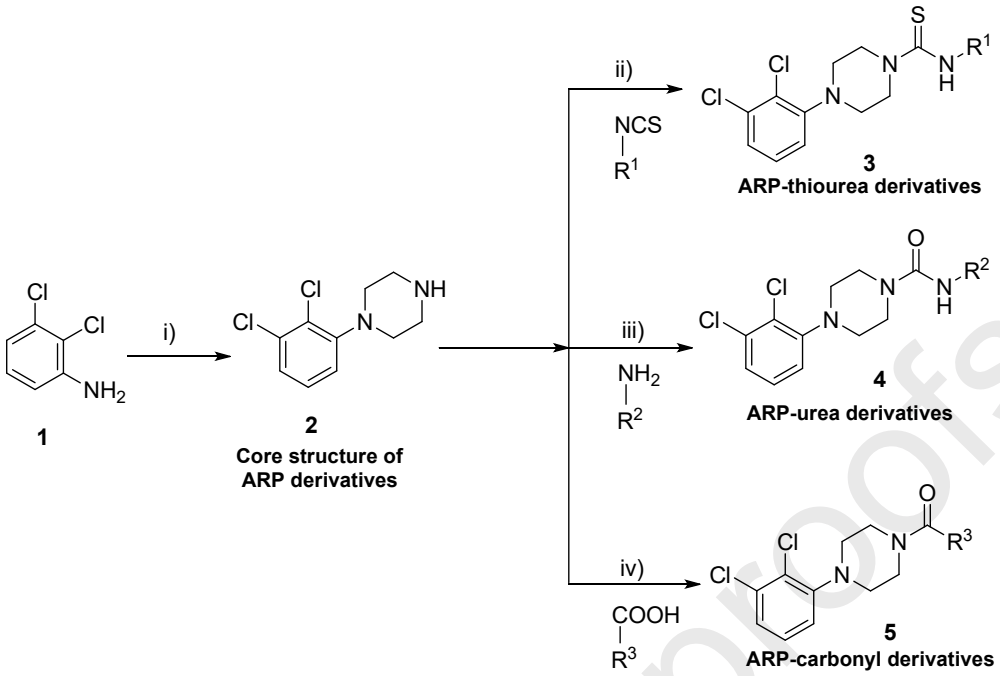


Figure 1. (a) Various bioactive small molecules embedded in an arylpiperazine (ARP) and a di-chloro ARP core structure. (b) Design strategy of ARP derivatives for type I IFN inducers with a different linker moiety.

Therefore, we aimed to develop new small-molecule regulators to stimulate type I IFN effect and identified that particular arylpiperazine (ARP) derivatives exhibited the desired biological activity using phenotypic screening. Remarkably, the ARP core structure was reported to exert anti-malarial, anti-microbial, anti-cancer activity or selective 5-HT_{1A} antagonistic effect (Figure 1a) [17–20]. In addition, 2,3-di-chloro substituent in ARP pharmacophore plays important role in particular drugs such as cariprazine, aripiprazol or bioactive DRD3 antagonist (Figure 1a) [21–23]. Based on these important characteristics of the ARP skeleton, we designed and synthesized a series of 2,3-dichloro ARP derivatives by further *N*-modification on piperazine part with different linker moiety such as thiourea, urea, and carbonyl functional group in order to assess structure-activity relationship (SAR; Figure 1b), then we evaluated their biological activities associated with the immune response.



Scheme 1. Synthesis of Arylpiperazine derivatives.[a]

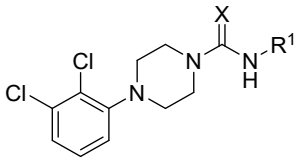
[a]Reaction conditions and reagents: (i) bis(2-chloroethyl)ethylamine, PTSA, tetrabutylammonium bromide, xylene, 135 °C, 48 h, reflux, NH_4OH, (yield 88 %); (ii) triethylamine, DCM, 15 min, room temperature, (yield 85-95%).; (iii) Triphosgene, triethylamine, DCM, room temperature, 1 h, (yield 85-95%); (iv) HATU, DIPEA, dry DMF, room temperature, 12 h, (yield 85-90%).

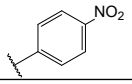
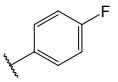
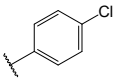
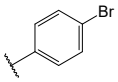
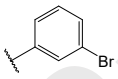
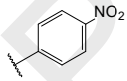
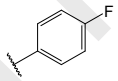
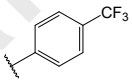
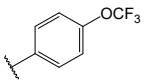
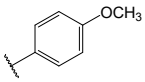
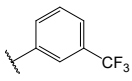
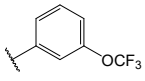
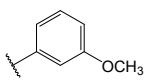
The series of ARP derivatives (**3**, **4**, and **5**) was completed in a two-step approach, as shown in Scheme 1. In the first step, 2,3-dichloroaniline (**1**) reacted with bis(2-chloroethyl)ethylamine to obtain 1-(2,3-dichlorophenyl) piperazine hydrochloride. Subsequently, 1-(2,3-dichlorophenyl)piperazine hydrochloride in the presence of NH_4OH was converted to a free amine ($-NH_2$) compound, 1-(2,3-dichlorophenyl)piperazine (**2**). The crude product **2** was modified with diverse substituents based on thiourea (**3**), urea (**4**), and a carbonyl linker (**5**). For instance, derivatives **3a** to **3f** were produced from the reaction between **2** and various thiocyanates with triethylamine (TEA) in dry DCM. The intermediate **2** reacted with aromatic amines in the presence of triphosgene (1.5 equiv.) to produce compounds **4a** to **4h** (see Supplementary material). The amide coupling reaction between **2** and acid derivatives (1 equiv.) in presence of HATU and DIPEA (3 equiv.) in DMF produced compounds **5a** to **5k** (see Supplementary material). Most of the compounds occurred at moderate yield (85%–90%) and were characterized by low-resolution mass spectroscopy, ^{1}H NMR, and ^{13}C NMR. Purity of the compounds was analyzed using high-performance liquid chromatography (HPLC) before performing biological

assays.

All the synthesized compounds were evaluated using ISRE reporter assay on THP-1 human monocyte cells for monitoring immune response. In this system, ISG54 minimal promoter in conjunction with five ISRE elicits transcription of secreted luciferase reporter gene upon stimulation of the type I IFN pathway, and IFN-related immune activation was measured based on luminescence [24].

Table 1. ISRE reporter assay of ARP derivatives with thiourea or urea linker.



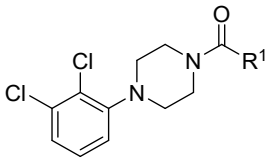
Compound	X	R^1	RU[a]	Cell viability (%)[b]
3a	S		1.2 ± 0.4	84 ± 15
3b	S		1.1 ± 0.3	97 ± 2
3c	S		1.3 ± 0.3	81 ± 7
3d	S		1.2 ± 0.2	99 ± 4
3e	S		0.4 ± 0.2	46 ± 54
3f	S		1.5 ± 0.2	85 ± 9
4a	O		1.0 ± 0.3	90 ± 10
4b	O		2.1 ± 1.7	95 ± 11
4c	O		3.9 ± 1.4	61 ± 16
4d	O		4.2 ± 1.3	96 ± 11
4e	O		3.3 ± 2.0	98 ± 16
4f	O		3.3 ± 1.1	76 ± 17
4g	O		3.0 ± 1.3	70 ± 26
4h	O		2.7 ± 1.0	87 ± 15

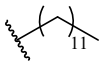
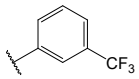
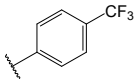
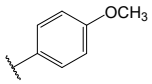
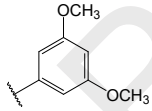
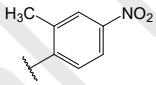
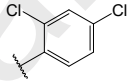
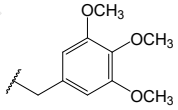
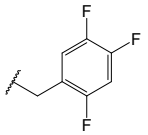
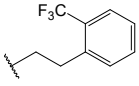
[a] Relative unit of ISRE reporter signal. Normalized response using DMSO as a control. [b] THP-1 cell viability. RU and cell viability were measured by treatment with 40 μM of each compound and are shown as mean ± standard deviation.

As a result of the SAR study, all thiourea derivatives revealed negligible or very weak potency, regardless of alkyl and aryl moiety at R^1 (**3a** to **3f**). No significant effects of *p*-nitro, fluoro, chloro substituents were observed on the aryl ring, however, the *p*-bromo substitution revealed undesired cellular toxicity (**3c** to **3e**). In the aryl part, *meta* substitution lead to slightly increased activity compared to *para* substitution (**3e** and **3f**).

To enhance activity, we introduced a urea moiety as a linker with diverse substitution on the aryl ring, which increased potency of ARP analogues for immune stimulation. Compared with the thiourea linker, *para* substitution on the phenyl ring exhibited stronger potency than *meta* substitution in most urea linker cases (**4a** to **4h**). In *para*-substituted derivatives, no significant activity was observed in the nitro moiety (**4a**) which corresponded to thiourea linker, whereas the fluoro and trifluoromethyl moieties showed increased activity (**4b** and **4c**). However, compound **4c** showed undesired cellular toxicity, thereby a trifluoromethyl substituent was replaced by a trifluoromethoxy moiety which substantially enhanced potency (4.2-fold change) and generated no toxicity (**4d**).

Table 2. ISRE reporter assay of ARP derivatives with carbonyl linkers.



Compound	R^1	RU[a]	Cell viability (%)
5a		1.3 ± 0.7	89 ± 16
5b		2.5 ± 0.8	89 ± 8
5c		2.8 ± 0.3	105 ± 20
5d		1.8 ± 0.1	90 ± 15
5e		3.7 ± 0.9	81 ± 16
5f		1.9 ± 1.1	83 ± 14
5g		1.4 ± 0.6	84 ± 16
5h		2.3 ± 1.2	86 ± 18
5i		5.2 ± 1.5	98 ± 22
5j		2.8 ± 1.7	101 ± 15
5k		2.5 ± 1.4	96 ± 12

[a] Relative unit for ISRE reporter signal. Normalized response by DMSO as a control. [b] THP-1 cell viability. RU and cell viability were measured by treatment with 40 μM of each compounds and are shown as mean ± standard deviation.

Considering the interesting results of thiourea and urea linker, we next incorporated carbonyl moiety as a linker for ARP derivatives. Somewhat weaker or stronger potency was observed in case of trifluoromethyl and methoxy substituents, compared to individual functional group in urea linker (**5c** to **5e**). For the further investigation, aliphatic chain or heteroaryl group were introduced at R^1 position which produced no significant difference and only marginally increased activity (**5a** and **5b**). Furthermore, disubstituted ARP analogues were considered to increase activity (**5f** to **5h**) that resulted in weak activity which was below that of *p*-methoxy analogue (**5e**).

To increase potency of ARP derivatives, we examined the effects on carbon element by incorporating a benzyl group with a trisubstituted functional group. For this purpose, trimethoxy benzyl (**5i**) or tri-fluoro benzyl (**5j**) were introduced at the phenyl ring. As a result, immune response was considerably improved due to tri-methoxy substitution on the aryl ring (5.2-fold change), whereas tri-fluoro benzyl substitution showed no drastic change on ISRE result with a bit increase of activity (2.8-fold change). In addition, we confirmed no effect on cell viability by benzyl group. For the further modification, we inserted a phenethyl moiety at R^1 position (**5k**) to assess effects of carbon chain which showed activity similar to that of dichlorophenyl (**5h**) or trifluorobenzyl (**5j**) moiety (2.5, 2.3 and 2.8, respectively) Based on these SAR results, we identified the compound **5i** as an effective type I IFN inducer and conducted further biological evaluation.

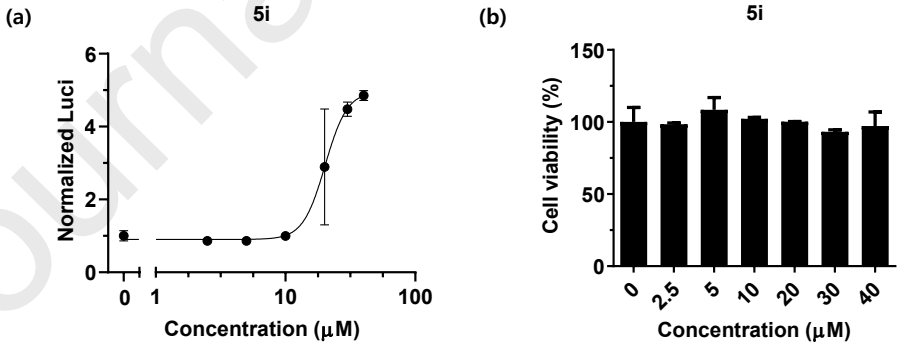


Figure 2. (a) Dose-response curve of the ISRE reporter assay. (b) THP-1 cell viability in **5i** treatments.

To estimate the potency of compound, we tested dose-dependency of immune responses elicited by **5i,** which clearly induced stimulation of ISRE reporter signal. EC_{50} was calculated as 13.1 μM and E_{max} was interpolated as 4.9-fold change (Figure 2a). No cellular toxicity to THP-1 cells was observed at treatment dosages of up to 40 μM (Figure 2b).

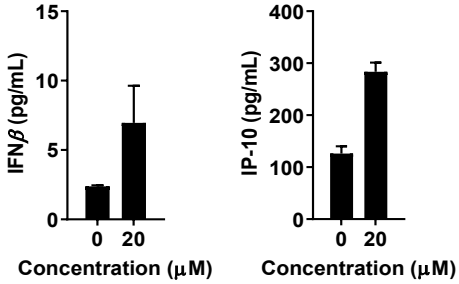


Figure 3. Evaluation of the effect on IFNβ and IP-10 secretion by compound **5i**. Cytokine levels were measured using ELISA.

We further evaluated whether **5i**-mediated activation of ISRE reporter signal would be correlated with activation of innate immunity. Using ELISA assay, we measured extracellular release of IFNβ and IP-10 which are associated with activation of the type I IFN pathway. The results confirmed that **5i** certainly induced secretion of IFNβ and IP-10 (Figure 3).

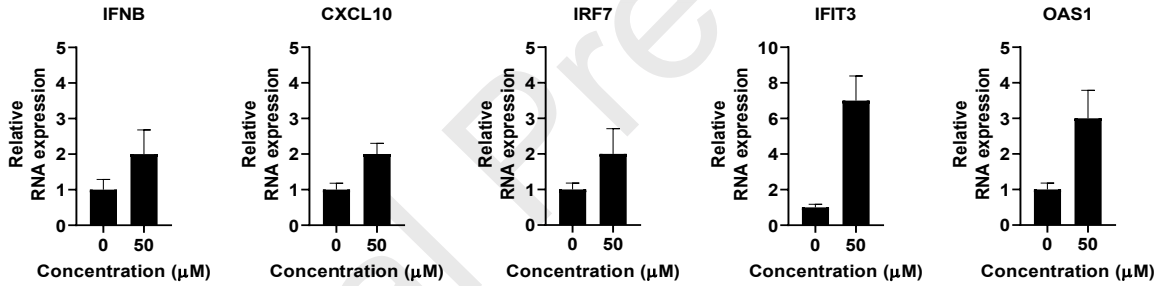


Figure 4. Gene expression results of various interferon-stimulated genes. mRNA expression level of IFNB, CXCL10, IRF7, IFIT3, OAS1 was examined by qPCR and was normalized using GAPDH expression. Graph are shown as the average value and RQmax.

In response to virus infection, host cells released various cytokines such as IFNs and the cytokine-mediated anti-viral status is established and maintained by production of various ISGs. To validate effects of **5i** on anti-viral state, we explored the impact of **5i** on type I IFN-induced ISG expression. Corresponding to the cytokine level, mRNA expression of IFNB and CXCL10 was clearly increased by **5i** treatment (Figure 4). In addition to IFNB and CXCL10, **5i** significantly elicited transcription of various ISGs such as IRF7, IFIT3, and OAS1 which are considered biomarkers of type I IFN signaling pathway (Figure 4). In conclusion, all these results indicated the potential ability of **5i** as an efficient type I IFN inducer without undesired toxicity. Although, it is hard to conclude that **5i** showed superior potency

compared to tilorone, an efficient small molecule anti-viral agent inducing type I IFN, due to the different evaluating system. Considering 50 mg/kg required to treat Evola virus by tilorone [25], the fact that **5i** induced significant increased IFNβ secretion at 20 μM suggested structure insight for developing new anti-viral therapy.

In conclusion, we confirmed ARP motif as a new core skeleton of type I IFN regulation. Moreover, we synthesized various ARP analogues and investigated their biological activity for anti-viral state and innate immunity. Based on the SAR analysis, we verified the importance of the carbonyl linker and the benzyl moiety with a trimethoxy attachment on adjacent aryl ring in the ARP pharmacophore. As a result, compound **5i** was identified as a potent type I IFN inducer. Further investigation about cytokine secretion and IFN-mediated ISG expression by **5i** indicated effective stimulation of type I IFN pathway. All these results suggest a beneficial therapeutic potential of **5i** for anti-viral agent.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplemental data

Supplementary data to this article including detailed experimental procedures can be found online

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Declaration of interests

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