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DOI: 10.1039/C6MD00427J

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RESEARCH ARTICLE

Design, synthesis and phenotypic evaluation of *N*-biaryl amides for IL-17A suppression†

Received 00th xxx 20xx,
Accepted 00th xxx 20xx

DOI: 10.1039/x0xx00000x

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A series of *N*-biaryl amides for Th17 cell phenotypic screening was designed and quickly prepared by microwave-assisted solid-phase organic synthesis. Structure-activity relationship of the three regions of the amides was explored and potent small molecular inhibitors to suppress IL-17A production in mouse Th17 cells were identified. Compound **6h** showed excellent suppression of IL-17A with an IC_{50} of 13 nM, which can be employed as a chemical tool for target identification and Th17 cell-related biology studies.

The interleukin 17 (IL-17) family, a subset of cytokines consisting of IL-17A–F, plays important roles in host defence against microbial organisms and in the development of inflammatory diseases.[1] The most widely studied cytokine of this family, IL-17A, is a pro-inflammatory cytokine which has potential effect in various inflammatory conditions such as autoimmune diseases, metabolic disorders, and cancer.[2-4] Current clinical phase III trials of IL-17A inhibitors such aslxekizumab (LY2439821), brodalumab (AMG-827), and the recently approved drug secukinumab (AIN457), have shown promising results as the proof of concept.[5] However, synthetic molecules as ligands for IL-17A/IL-17R have not been found yet, indicating that IL-17A/IL-17R is a challenging target for discovery of small molecule inhibitors.

T helper 17 (Th17) cells, a subset of $CD4^+$ T helper cells, are one of the major sources of IL-17A in a variety of human autoimmune inflammatory diseases.[6] Th17 cells can be differentiated from naïve $CD4^+$ T cells in the presence of TGF-β and IL-6, which activates downstream signalling factors including STAT3 and the orphan nuclear receptor RORγt. Other cytokines such as IL-1, IL-21 and IL-23 have also been shown to be able to promote Th17 cell differentiation in pathological conditions.[7-13] Accordingly, Th17 cell differentiation can be considered as an exploitable cellular phenotype.

RORγt is a key transcription factor regulating Th17 cell differentiation and IL-17A production.[14] Recently we reported discovery of a series of novel small molecule RORγt inverse agonists (e.g., **1** in Fig. 1).[15-18] RORγt activity was measured by the RORγ FRET peptide recruitment assay.[19] Subsequent biological evaluation of synthetic compounds was carried out by the Th17 cell differentiation assay, in which IL-17A production was measured for the validation of the compounds activity at cellular level.[15] During the structure activity relationship (SAR) exploration, we noticed that a set of *N*-biarylbenzamides (e.g., **2**) showed no RORγt activity in the FRET assay but had reasonable activity in Th17 cell differentiation assay (e.g., 39% of IL-17A inhibition@0.3μM for **2**). No activity of **2** in the RORγ FRET assay indicated that such suppression activity of IL-17A was unlikely caused by inverse agonism of RORγt. Due to the positive effect of inhibiting IL-17A production and easy modification/diversification, we hypothesized that *N*-biaryl amides have the potential to be developed into an IL-17A-suppressive small molecular lead using Th17 cell differentiation assay as cellular phenotypic screening strategy. In this paper, we report design, rapid synthesis and phenotypic evaluation of a series of *N*-biaryl amides as potential suppressors of IL-17A production by Th17 cells.

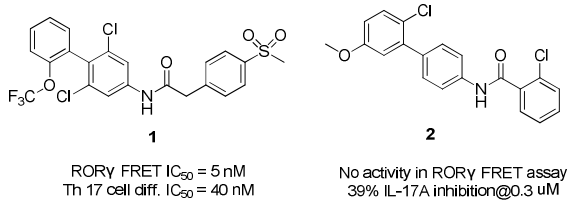

Fig. 1. RORγt inverse agonist **1** and IL-17A suppressive hit **2**

Based on structure of **2**, we designed a small library of *N*-biaryl amides (**3** in Fig. 2) with right hand side (RHS) R, middle Ar_1 and left-hand side (LHS) Ar_2 as variables. While Ar_1 and Ar_2 are aromatic moieties, R can be aromatic or non-aromatic moieties. In order to explore SAR of each component, three

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† The authors declare no competing interests.
Electronic Supplementary Information (ESI) available: All the experimental details, NMR spectra and LC-MS analysis. See DOI: 10.1039/x0xx00000x

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DOI: 10.1039/C6MD00427J

sets of compounds (**4**, **5**, **6**) with one variable at one time were designed for synthesis and biological evaluation.

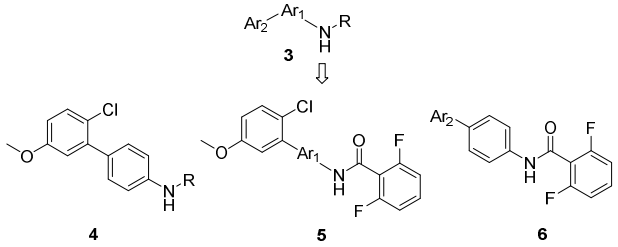
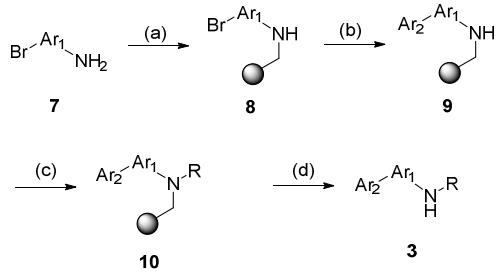


Fig. 2. General structure**3** and SAR Sets **4**-**6**of *N*-biaryl amides

Solid phase organic synthesis (SPOS) has been a powerful tool for the quick preparation of compound libraries used for various types of screenings and hit to lead/lead optimization in drug discovery.[20] Microwave-assisted organic synthesis (MAOS) is being also widely used in reaction optimization and solution-phase parallel synthesis mainly due to its ability to shorten reaction time.[21] Microwave-assisted solid phase organic synthesis (MASPOS), combining advantages of SPOS and MAOS, not only fulfils green chemistry's needs (environmental friendly– energy efficiency and less organic waste), but enables us to efficiently prepare SAR compounds containing various functional moieties related to a similar scaffold for hit to lead optimization.

A general synthetic route for preparing library **3** was shown in Scheme 1. Bromo aryl amines **7** were loaded into 2,6-dimethoxy-4-polystyrenebenzyloxy-benzaldehyde resin (DMHB resin) though reductive amination.[22] The Suzuki reaction of the resulting resin-bound bromides **8** and various boronic acids under microwave conditions produced resin-bound biaryl amines **9**. The resin-bound amines **9** reacted with different carboxylic acids formed the resin-bound *N*-biaryl amides **10**, which upon acidic cleavage of resin yielded the desired *N*-biaryl amides **3**. Initially, 21 carboxylic acids, 5 bromo aryl amines and 10 aryl boronic acids were selected for library synthesis.

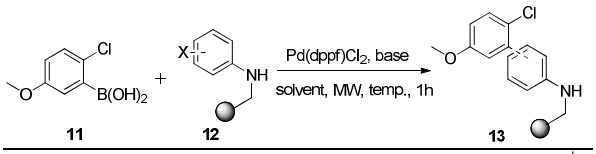


Scheme 1. General synthesis of *N*-biarylamides[a]

[a] Reagents and conditions: (a) DMHB-resin, $Na(OAc)_3BH$, DIPEA, 15% HOAc in NMP, rt; (b) various aryl boronic acids, $Pd(dppf)Cl_2$, $K_3PO_4{\cdot}3H_2O$, NMP, microwave radiation, 90 °C, 1h; (c) various carboxylic acids, DIC, DMF:DCE (1:1, v/v), microwave radiation, 60 °C, 1h; (d) 50% TFA in DCE, 30min, rt.

The Suzuki reaction is the key step of the whole synthetic route and its reaction conditions had to be optimized in order to get high conversion and purity of product for the next step. $Pd(dppf)Cl_2$ was selected as the catalyst and other conditions such as solvent (NMP), base ($K_3PO_4{\cdot}3H_2O$) and microwave reaction temperature (90°C) were chosen based on the reaction results shown in Table 1. A typical experimental procedure for preparation of *N*-biaryl amides was described using **6h** as an example.[23]

Table 1. Optimization of the Suzuki reaction conditions[a]



Entry	X	Solvent	Base	Temp. (°C)	Yield[b] (%)
1[c]	3-Br	DMF	Na_2CO_3	110	92
2[c]	3-Br	DMA	Na_2CO_3	110	91
3	3-Br	NMP	Na_2CO_3	110	90
4	3-Br	PEG_{400}:H_2O (1:1)	Na_2CO_3	110	4
5	3-Br	NMP	K_2CO_3	110	95
6	3-Br	NMP	$K_3PO_4{\cdot}3H_2O$	110	100
7	3-Br	NMP	Cs_2CO_3	110	89
8	4-Br	NMP	$K_3PO_4{\cdot}3H_2O$	110	72
9	4-Br	NMP	$K_3PO_4{\cdot}3H_2O$	100	89
10	4-Br	NMP	$K_3PO_4{\cdot}3H_2O$	90	100
11[c]	4-Br	NMP	$K_3PO_4{\cdot}3H_2O$	80	70

[a]Reaction Conditions: 11 (96.9mg, 0.52mmol), 12 (100mg, 0.13mmol), $Pd(dppf)Cl_2$ (14.3mg, 0.02mmol), base(0.39mmol), 1mL solvent bubbled with nitrogen, microwave radiation at appointed temperature.
[b]Yield was based on LC-MS relative peak areas monitoring at 254nm.
[c]By-product existed.

A total of 34 compounds (21 from Set **4**, 4 from Set **5** and 9 from Set **6**) were screened in mouse primary Th17 cell differentiation assay at concentration of 0.3 μM.[24] Percentages of maximum inhibitions for each set of compounds were summarized in Tables 2-4.

The RHS SAR of the amides was shown in Table 2. Substitution on ortho-position of the phenyl ring (**4b**, **2**, **4c**) slightly influenced the suppressive effect on IL-17A production with F >Cl > CF_3. Replacing phenyl (**4b**) with pyridine (**4d**) lowered the activity. Interestingly, 2,6-di-substitution on the phenyl

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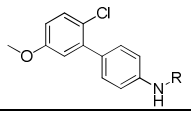
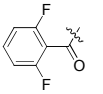
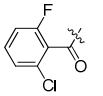
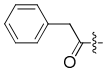
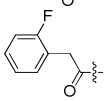
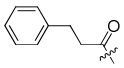
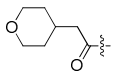
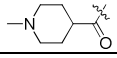
ring (**4e** and **4f**) displayed much enhanced inhibitory effect compared to no or single ortho-substitution. Inserting one or two CH_2 between phenyl ring and carbonyl group (**4g-4i**) decreased potency to inhibit IL-17A production. Changing the phenyl ring to cycloalkyl (**4j**, **4n**, **4p**) or hetero-cycloalkyl (**4k**, **4m**, **4o**) did not improve the inhibitory activity. Other non-aromatic groups (**4l**, **4q**-**4t**) decreased the inhibitory effect to IL-17A production.

Table 2. RHS SAR of the amides

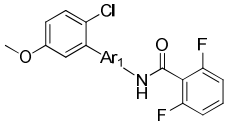


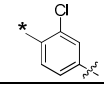
Compd	R	IL-17A inh.%@0.3μM[a]
4a		35
4b		43
2		39
4c		25
4d		22
4e		71
4f		64
4g		33
4h		19
4i		24
4j		26
4k		35
4l		8
4m		16
4n		34
4o		30
4p		35
4q		9
4r		22
4s		1
4t		11

[a]All results are an average of at least n=2.

Fixing the RHS moiety as 2,6-difluoro-phenyl, SAR of middle aryl in *N*-biaryl amides was summarized in Table 3. 1,4-Substituted phenyl (**4e**) showed much better effect on inhibiting IL-17A production than the 1,3-substituted phenyl (**5a**). Small substituents such as F (**5c**) or Cl (**5d**) on the phenyl slightly increased their suppressive activity while pyridine ring (**5b**) replacing phenyl ring dropped the activity dramatically.

Table 3. Middle aryl SAR of the amides



Compd	Ar_1	IL-17A inh.%@0.3μM[a]
4e		71
5a		12
5b		29
5c		82
5d		75

[a]All results are an average of at least n=2.

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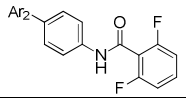
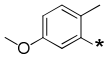
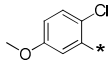
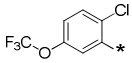
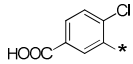
Table 4. LHS SAR of the amides



Compd	Ar_2	IL-17A inh.%@0.3μM[a]
6a		45
6b		17
6c		4
6d		-7
6e		60
6f		48
6g		93
4e		71
6h		97
6i		7

[a]All results are an average of at least n=2.

LHS SAR of the amides was shown in Table 4. Pyridines (**6b**, **6c**) and pyrazole (**6d**) dropped much of their effect on suppressing IL-17A production compared to the phenyl compound (**6a**). Substituents on ortho-position of the phenyl (**6e**, **6f**) enhanced the inhibitory activity on IL-17A production with Me > Cl. Putting additional methoxy group on 5-position of 2-Me-phenyl (**6g**) or 2-Cl-phenyl (**4e**) boosted their effects of inhibiting IL-17A production greatly. Replacing -OMe group (**4e**) with -OCF_3 group (**6h**) further enhanced the inhibitory activity with a maximum inhibition of 97%@0.3μM. However, switching the -OMe to a polar group like –CO_2H (**6i**) dropped the activity dramatically, indicating that a polar group at this position may impair the activity.

Since compound **6h** showed a strong inhibitory effect on IL-17A production by Th17 cells compared to the starting hit **2** (Fig. 3A), we performed a dose-dependent study on **6h** in mouse primary Th17 cell differentiation system. As showed in Fig. 3B, IL-17A inhibition for **6h** is dose-dependent with an IC_{50} of 13 nM. Like other compounds in the series, **6h** showed almost no RORγt activity (IC_{50} > 50 μM) in RORγ FRET assay, indicating that **6h** is not a RORγt inverse agonist.

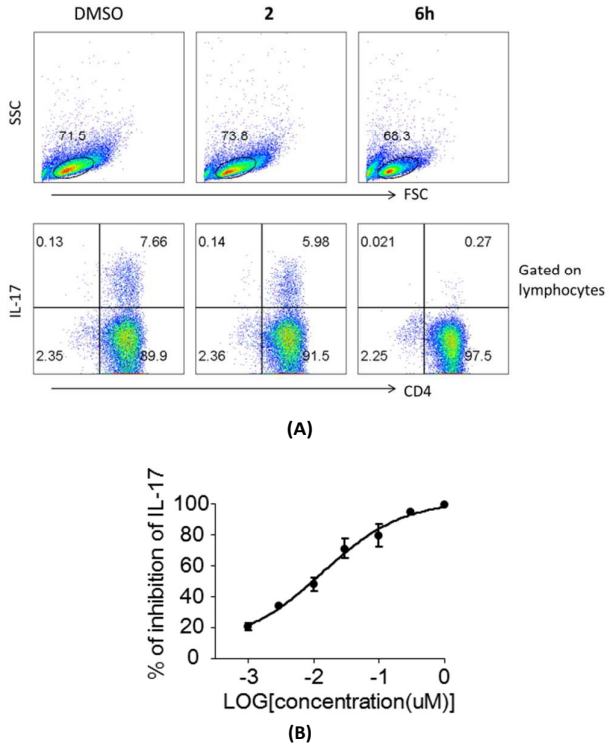


Fig. 3. (A) Flow cytometry analysis of IL-17A production in Th17 cells after treatment of DMSO, 0.3 μM compound **2** and 0.3 μM compound **6h**; (B) Dose-dependent study of **6h** in Th17 cell differentiation assay. Error bars represent SEM (n=3). Data are representative of 2 independent experiments.

In summary, we have designed and quickly prepared a series of *N*-biaryl amides for phenotypic screening of Th17 cells, characterized by IL-17A production, using microwave-assisted solid-phase organic synthesis. Structure-activity relationship of the three regions of the amides was explored and potent small molecular inhibitors to suppress IL-17A production in mouse primary cell were identified. Compound **6h** showed excellent suppression of IL-17A with IC_{50} of 13nM, which can be used as a chemical tool for target identification and Th17 cell-related biology studies.

Acknowledgements

This work was supported by Grant81573276 from National Science Foundation of China, Grant2015CB943400 and 2014CB943300 from the Ministry of Science and Technology of China, Grant 14140902400 from the Experimental Animal of Shanghai Science and Technology Committee, Grant91542102 and 31570887 from National Science Foundation of China, and China's Youth 1000-Talent Program to J. Q.

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23. Synthetic procedure of *N*-(2'-chloro-5'-(trifluoromethoxy)-[1,1'-biphenyl]-4-yl)-2,6-difluorobenzamide (**6h**). (1) To a 250mL flask were added 2,6-dimethoxy-4-polystyrenebenzyloxy-benzaldehyde (DMHB resin) (10g, 15mmol) and 100mL 1-methyl-2-pyrrolidinone (NMP) and swelled for 5 min. 4-Bromoaniline (13g, 75mmol), DIPEA (13mL, 75mmol) and acetic acid (15mL) were then added. After shaking at room temperature for 2 hours, sodium triacetoxyborohydride (19g, 90mmol) was added gradually and then the mixture continued to shake for 24 hours at room temperature. The loaded resin was washed with MeOH, DMF, H_2O, MeOH and DCM successively and dried in vacuum overnight to yield DMHB resin-bound 4-bromo-benzylamine. (2) To mixture of the above DMHB resin-bound 4-bromo-benzylamine (100mg, 0.13mmol) in 1mL NMP were added (2-chloro-5-(trifluoromethoxy)phenyl) boronic acid (125mg, 0.52mmol), $K_3PO_4 \cdot 3H_2O$ (104mg, 0.39mmol). After bubbled with nitrogen for 5 min, the mixture was added $Pd(dppf)Cl_2$ (14.3mg, 0.15mmol) and set up in microwave reactor at 90 ^oC, 1h, pre-stirring for 30 seconds. The resulting resin was washed with THF, THF:H_2O (1:1), H_2O, THF:H_2O (1:1), THF, DCM successively and dried in vacuum for 6 hours. An analytical amount of the resin was cleaved with 15% TFA in DCM for 10 min. The resulting solution was evaporated and basified by saturated sodium carbonate solution and extracted with 0.5mL DCM detected by LC-MS. LC- MS (ESI): 287.0 $[M+H]^+$. (3)To mixture of the above DMHB resin-bound 2'-chloro-5'-(trifluoromethoxy)-[1,1'-biphenyl]-4-amine (200mg, 0.25mmol) in 2mL DMF:DCE (1:1) were added 2,6-difluorobenzoic acid (389mg, 2.5mmol), *N,N'*-methanediylidenebis(propan-2-amine) (DIC) (381µL, 2.5mmol). The mixture was sealed in a tube and set up in microwave reactor at 60^oC, 1h, pre-stirring for 30 seconds. The resulting resin was washed with MeOH, DMF, H_2O, MeOH and DCM successively and dried in vacuum overnight, and then treated with 50% TFA in DCE for 30 min. The cleaved solution was concentrated and basified by saturated sodium carbonate solution and extracted with DCM. The combined organic phase was evaporated and isolated by PTLC to get the desired compound. LC-MS (ESI): 427.0 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.74 (d, J = 8.4 Hz, 2H), 7.57 – 7.35 (m, 4H), 7.22 (s, 1H), 7.16 (d, J = 8.7 Hz, 1H), 7.01 (t, J = 8.2 Hz, 2H)
24. Mouse Th17 differentiation assay and measurement of IC_{50}: $CD4^+$ T cells were purified from mouse splenocytes using a commercial $CD4^+$ T cell negative selection kit (Invitrogen). $CD4^+$ T cells were skewed to Th17 cells by culturing cells in the presence of anti-CD3(0.25ug/ml, Bioxcel), anti-CD28(1ug/ml, Bioxcel), anti-IFN-γ(2ug/ml, Bioxcel), anti-IL-4(2ug/ml, Bioxcel), TGF-β(5ng/ml, Peprotech) and IL-6(20ng/ml, Peprotech) for 4 days before analysis. Compounds or DMSO control were added

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to the culture on day 0 of Th17 differentiation at indicated concentrations. Percentage of IL-17A production from $CD4^+$ T cells were analyzed by intracellular staining followed by flow cytometry. Dose-response curves were plotted to determine half-maximal inhibitory concentrations (IC_{50}) for the compounds using the GraphPad Prism 5 (GraphPad Software, San Diego CA, USA). All experiments were performed in compliance with the relevant laws and institutional guidelines, and all experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Health Sciences, Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences.

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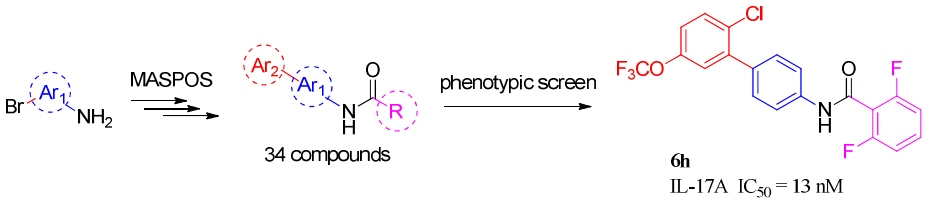
Graphical Abstract



A series of *N*-biaryl amides was designed, quickly prepared by microwave-assisted solid-phase organic synthesis and phenotypically evaluated via mouse Th17 cell differiation assay.