Bioorganic & Medicinal Chemistry 16 (2008) 8760–8764

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Salicylanilides: Selective inhibitors of interleukin-12p40 production

Michael E. Brown [a], Jeffrey N. Fitzner [a], Tracey Stevens [b], Wilson Chin [b], Clifford D. Wright [b], Jim P. Boyce [a,*]

[a] Medicinal Chemistry, Amgen Inc., 1201 Amgen Court West, Seattle, WA 98119, USA
[b] Inflammation, Amgen Inc., 1201 Amgen Court West, Seattle, WA 98119, USA

ARTICLE INFO

Article history:
Received 29 February 2008
Revised 30 June 2008
Accepted 14 July 2008
Available online 17 July 2008

Keywords:
Salicylanilide
Interleukin-12p40 (IL-12p40)
Interleukin-12 (IL-12)
Interleukin-23 (IL-23)
Interleukin-6 (IL-6)

ABSTRACT

Interleukin (IL)-12p40, a subunit component of both IL-12 and IL-23, is being widely studied for its role in inflammatory disease. As part of an effort to profile cellular signaling pathways across different cell types, we report salicylanilide inhibitors of IL-12p40 production in stimulated dendritic cells. Based on a hypothesis that a desirable therapeutic profile is one that could block IL-12p40 but not IL-6 production, we engaged in directed analoging. This resulted in salicylanilides with similar IL-12p40 related potency but enhanced selectivity relative to IL-6 production.

1. Introduction

1.1. Interleukin-12p40 biology

Interleukin (IL)-12p40 is a subunit component of both IL-12 and IL-23. IL-12 is involved in type 1 helper T cell (Th1) mediated inflammation in both normal immune defense as well as inflammatory diseases such as rheumatoid arthritis, asthma, psoriasis, and Crohn's disease.[1–5] IL-23 has a divergent immunological role in regulating T cells that produce other pro-inflammatory cytokines. IL-12p40 may exist in monomeric or homodimeric (IL-12p80) forms that have been recognized to possess their own bio-activities, independent of IL-12 and IL-23, and which include chemoattraction of macrophages and stimulated dendritic cells.[6]

1.2. Bio-activity of salicylanilides

As a class, salicylanilides have been reported with a wide variety of interesting biological properties.[7,8] For example, Efuamide, **1**, has been reported to be immunosuppressive in experimental allergic encephalomyelitis models in animals and to inhibit phosphorylation and signaling of erbB-2 resulting in cell cycle arrest.[9,10] Niclosamide, **2**, was originally discovered as a molluscicidal and was subsequently developed as an orally administered, broad spectrum anthelmintic[11,12] (see Fig. 1).

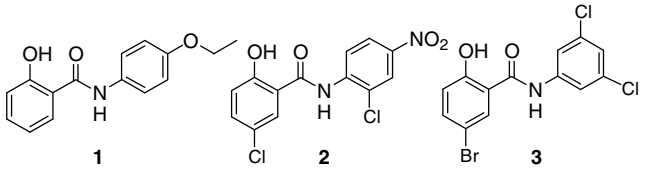


Figure 1. Examples of bio-active salicylanilides.

2. Results and discussion

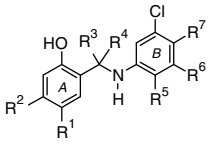
As a result of data analysis across an array of cell based assays, we found that salicylanilide **3** inhibited IL-12p40 and IL-6 production in stimulated primary human dendritic cells as well as behaving similarly in a dendritic cell line assay (Table 1).[13] Cytotoxicity profiling for compound **3** was assessed and did not explain the IL-12p40 functional activity. With respect to inhibition of IL-12p40 production, analog **4** indicated that the amide carbonyl moiety of **3** is important for IL-12p40 (and IL-6) activity.

Analog **5**, with a nitro-group instead of the bromo-group featured in **3**, resulted in a loss of activity toward IL-12p40 but little change in IL-6 activity, relative to **3**. A similar result was observed with the isomeric nitro-salicylanilide **6**. Compounds **7** and **8** demonstrate that other halogen substitution patterns, on the anilide ring (i.e. 'B'), are tolerated with respect to the IL-12p40 assay but still little or no selectivity was observed relative to the IL-6 assay. In comparison with compound **3**, analog **9**, in which there is a hydrogen in place of the bromine on the

* Corresponding author. Tel.: +1 425 821 6971; fax: +1 206 217 5529.
E-mail addresses: boycej@amgen.com, jboyce2000@aol.com (J.P. Boyce).

0968-0896/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.bmc.2008.07.024

Table 1
Inhibition of IL-12p40 production in stimulated dendritic cells



Entry	R1	R2	C(R3,R4)	R5	R6	R7	IC_{50} (μM)[a]	
							IL-12p40	IL-6
3	Br	H	C=O	H	Cl	H	0.013	0.30
4	Br	H	CH_2	H	Cl	H	2.7	1.7
5	NO_2	H	C=O	H	Cl	H	0.41	0.17
6	H	NO_2	C=O	H	Cl	H	0.32	0.25
7	Cl	H	C=O	H	H	Cl	0.13	0.13
8	Br	H	C=O	Cl	H	Cl	0.074	0.64

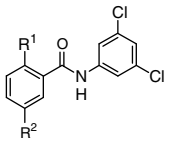
[a] Data reported here are for a murine dendritic cell line stimulated with a mixture of CD40L, IFNγ, and IL-18. IL-12p40 and IL-6 detection by fluorescence immunoassay.

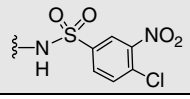
salicyl-ring (i.e. ring 'A'), was found in our assays to behave similarly to compound **3** with respect to inhibition of IL-12p40 production but to be much more selective against inhibition of IL-6 production (Table 2).[14]

We were interested in understanding the importance of the phenol group and so analogs featuring other functional groups, in place of the phenol, were prepared. We initially looked at *O*-alkyl ethers, such as the *O*-benzyl ether **10**, but these generally showed dramatic loss of inhibition of IL-12p40 production at our highest assay concentration (i.e. 10 μM). A promising exception to this, which is noted here and will be reported on more fully elsewhere, is the related, non-salicylanilide compound **11** in which a sulfonamide group, with a pK_a roughly close to that of a phenol, was incorporated on the benzoyl ring.[15] Halogen substitution on the anilide ring was generally favorable for IL-12p40 activity. The *meta*-chloro substituents of **9** are important for IL-12p40 activity and appear to have an additive effect when compared with compound **12** (Table 3). However, alternative halogen substitution patterns, such as featured in analog **13** can result in comparably active analogs. Hydrogen bond donor or acceptor groups, such as appear in analogs **14** or **15**, showed no inhibition of IL-12p40 production.

Comparison of **9** with **17**, in which the inductively electron-withdrawing chlorine substituents at the *meta* position of the anilide ring of **9** are replaced with isosteric methyl groups, resulted in complete loss of inhibitory activity in the IL-12p40 assay. The middling activity of analog **18** suggests an additional resonance (or subtle steric) effect that favors chlorines over trifluoromethyl groups at these *meta*-anilino positions (i.e. **9** vs **18**).

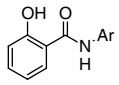
Table 2
Inhibition of IL-12p40 production in stimulated dendritic cells

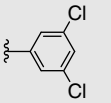
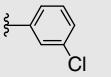
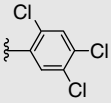
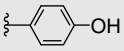
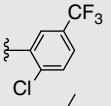
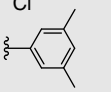
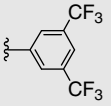


Entry	R1	R2	IC_{50} (μM)[a]	
			IL-12p40	IL-6
3	OH	Br	0.013	0.30
9	OH	H	0.013	>10
10	OBn	H	>10	>10
11		H	0.24	0.22

[a] See Table 1 comments for the IL-12p40 and IL-6 assays.

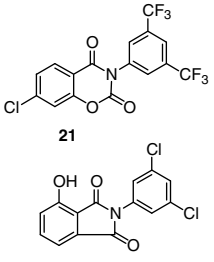
Table 3
Inhibition of IL-12p40 production in stimulated dendritic cells



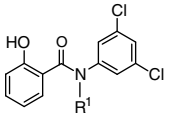
Entry	Ar	IC_{50} (μM)[a]	
		IL-12p40	IL-6
9		0.013	>10
12		5.1	>10
13		0.045	>10
14		>10	>10
15		>10	>10
16		0.20	1.8
17		>10	>10
18		0.12	0.74

[a] See Table 1 comments for the IL-12p40 and IL-6 assays.

Table 4
Inhibition of IL-12p40 production in stimulated dendritic cells



21



22

Entry	R1	IC_{50} (μM)[a]	
		IL-12p40	IL-6
9	H	0.013	>10
19	CH_3	>10	>10
20	CH_2CO_2H	>10	>10
21		0.28	0.35
22		>10	>10

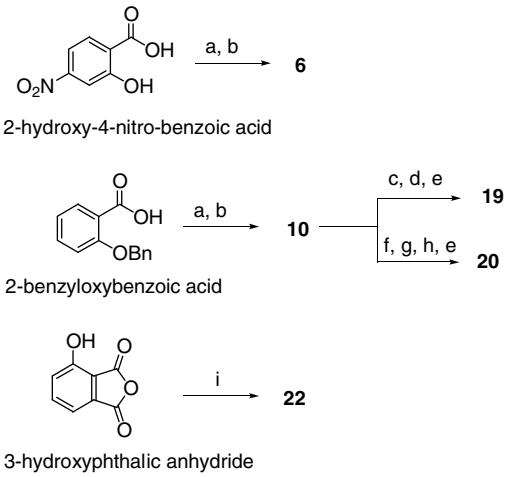
[a] See Table 1 comments for the IL-12p40 and IL-6 assays.

Lastly, simple substitution on the secondary amide nitrogen of **9** is not tolerated as evidenced by analogs **19** and **20** (Table 4). Interestingly, the benzoxazinedione **21**, which does not feature a secondary amide or an acidic phenol group but which bares some intriguing resemblance to these salicylanilide inhibitors, possessed some moderate IL-12p40 activity in our assay but also inhibited IL-6 production. In contrast, the phthalimide **22**, which might also be considered to be a conformationally constrained analog of **9**, lacked any inhibition of IL-12p40 production.

Broad counter-screening of **9** against a diverse collection of recombinantly expressed proteins showed no significant inhibition versus any of over 150 known and recombinantly expressed protein targets.[16]

3. Conclusions

We have reported that salicylanilides, such as **9**, can potently and selectively inhibit IL-12p40 production in dendritic cells. Further elucidation of the mechanism of action, of the compounds described here, will be reported.



Scheme 1. Example syntheses of salicylanilides. Reagents: (a) DCC, *N*-hydroxysuccinimide, THF; (b) 3,5-dichloroaniline; (c) NaH, THF; (d) CH_3I (e) Pd/C, H_2; (f) NaO-*t*-butyl, 1,2-dimethoxyethane; (g) *tert*-butyl bromoacetate; (h) TFA, CH_2Cl_2; (i) 3,5-dichloroaniline, AcOH, reflux.

4. Experimental methods

4.1. General synthesis methods

Unless noted otherwise, hydrogenations were performed using a Parr 3911 hydrogenation apparatus and microwave irradiations were conducted with a CEM Explorer microwave apparatus with auto-sampler. Chromatography-grade, anhydrous solvents were used without further purification. Concentration refers to distillation using a rotary evaporator (Buchi R-124). Flash chromatography was performed on E. Merck #60, 230–400 mesh silica gel. TLC analyses were performed using E. Merck glass backed silica gel #60 plates, 0.25 mm thickness, with 254 nm fluorescent indicator with visualization by UV absorption, iodine chamber, or solutions of $KMnO_4$ or other common staining techniques. NMR spectra were collected with a Varian Inova AS500 equipped with a Zymark auto-sampler and spectral resonance shifts are reported after internal standardization to a characteristic resonance shift of the solvent. Coupling constants refer to apparent peak multiplicities and abbreviations used are s = singlet, d = doublet, t = triplet, q = quartet, br = broad. HPLC analyses were performed on an Agilent 1100 Series HPLC, equipped with a reverse phase column (Zorbax Rx-C8, 9.4 mm ID × 25 cm) and using an elution gradient composed of increasing ratios of 0.1% v/v TFA in CH_3CN: 0.1% v/v TFA in H_2O with monitoring of analytes by UV absorption, evaporative light scattering (SEDEX ELSD) and mass spec. detection (ESI/CI). Mass spectra were collected on an Agilent 1100 MSD and are reported as percent intensity relative to the base peak. Except where noted otherwise, all reagents, including compound **14** [526-18-1], were purchased from Sigma–Aldrich Chemical Co.

Compounds **3** [439145-04-7], **4** [298215-62-0], **7** [642-84-2], **8** [254106-36-0], **9** [78154-58-2], **10** [882158-06-7], **13** [106480-61-9], **15** [13563-04-7], **16** [5683-92-1], **17** [2819-59-2], and **21** [284664-73-9] were purchased from Maybridge-Thermo Fisher Scientific Inc. Compound **11** [454691-62-4] was prepared according to the general method of Thomas and Allanson and compound **12** [24448-71-3] was prepared according to the general method of Liechti et al. and compounds **5** and **18** [744-58-1] were prepared according to the general method of Muto et al.[17–19] The following are synthesis protocols for new compounds **6**, **19**, **20**, and **22** (Scheme 1).

4.2. Experimental syntheses

4.2.1. *N*-(3,5-Dichlorophenyl)-2-hydroxy-4-nitrobenzamide (6)

A solution of 2-hydroxy-4-nitro-benzoic acid (2.34 g, 12.8 mmol), *N*-hydroxysuccinimide (1.48 g, 12.8 mmol), and *N,N′*-dicyclohexylcarbodiimide (2.68 g, 13.0 mmol) in THF (25 mL) was stirred (rt, 3 h), and the resulting precipitate was filtered and the filtrate concentrated in vacuo to afford a crude succinate ester (2.97 g) which was used without further purification: MS (−EI) *m/z* 279 (M^-, 100). A portion of this material (1.52 g, theor. 6.44 mmol) and 3,5-dichloroaniline (2.06 g, 12.7 mmol) was briefly warmed (140 °C, internal, 10 min) as a melt, with stirring, then allowed to cool (rt). After aqueous work-up (EtOAc/10% aq HCl), the organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo to afford a crude solid from which an analytical sample of **6** (130 mg, 0.397 mmol) was prepared by flash chromatography (hexanes/ethyl acetate, 17:3). ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.71 (br s, 1H), 10.75 (br s, 1H), 7.89 (d, J = 10.0 Hz, 1H), 7.84 (s, 2H), 7.77 (d, J = 10.0 Hz, 2H), 7.37 (s, 1H); MS (−EI) *m/z* 327 ((M^-, 70), 325 (M^-, 100).

4.2.2. *N*-(3,5-Dichlorophenyl)-2-hydroxy-*N*-methylbenzamide (19)

A solution of 2-benzyloxybenzoic acid (2.00 g, 8.76 mmol), *N*-hydroxysuccinimide (1.21 g, 10.5 mmol) and *N,N′*-dicyclohexylcarbodiimide (1.99 g, 9.64 mmol) in anhydrous THF (30 mL) was stirred (rt, 4 h). The resulting precipitant was filtered and the filtrate was concentrated in vacuo to afford *N*-hydroxysuccinyl 2-(benzyloxy) benzoate as a crude intermediate that was used without further purification (3.39 g): ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.93 (d, J = 7.5, 1H), 7.73 (m, 1H), 7.50 (d, J = 7.5 Hz, 2H), 7.38 (m, 3H), 7.32 (d, J = 7.0 Hz, 1H), 7.16 (m, 1H), 5.30 (s, 2H), 2.89 (s, 4H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.4, 160.2, 158.8, 136.4, 136.4, 131.7, 128.4, 127.7, 127.0, 120.8, 114.5, 113.9, 69.8, 25.5. An aliquot of this crude succinate ester (1.00 g, 3.07 mmol) and 3,5-dichloroaniline (1.50 g, 9.23 mmol) was briefly warmed as a melt (10 min, 140 °C, internal). The resulting yellow solution was allowed to cool (rt). The residue was diluted with EtOAc (100 mL) and after aqueous washing (3 N HCl, brine), the organic layer was dried ($MgSO_4$) and concentrated in vacuo to afford a tan solid which after crystallization (EtOAc) afforded 2-(benzyloxy)-*N*-(3,5-dichlorophenyl)benzamide **10**, CAS Registry [882158-06-7], as a white crystalline solid (0.67 g, 1.80 mmol): ^{1}H NMR (500 MHz,

$CDCl_3$) δ 10.09 (br s, 1H), 8.29 (d, J = 7.5 Hz, 1H), 7.54–7.52 (m, 6H), 7.15–7.12 (m, 2H), 7.08 (s, 2H), 7.97 (s, 1H), 5.19 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.0, 156.7, 140.2, 134.9, 134.8, 133.8, 132.6, 129.8, 129.3, 129.0, 123.5, 122.0, 120.9, 117.6, 112.5, 72.0; MS (+EI) m/z 374 (MH^+, 70), 372 (MH^+, 100). To a cold (0 °C bath) suspension of **10** (100 mg, 0.269 mmol) and NaH (60% w/w oil disp., 16 mg, theor. 0.4 mmol) in anhydrous THF (2 mL) was added neat methyl iodide (0.2 mL, 3 mmol) and the resulting mixture was allowed to warm (rt, 30 min) with stirring. The resulting mixture was carefully quenched with methanol (0.5 mL), filtered through a plug of silica gel (hexanes/EtOAc, 5:1), and concentrated in vacuo to afford 2-(benzyloxy)-*N*-(3,5-dichlorophenyl)-*N*-methylbenzamide as a colorless oil (91 mg, 0.24 mmol) and which was used without further purification: MS (+EI)*** m/z 388 (MH^+, 70), 386 (MH^+, 100). A suspension of 2-(benzyloxy)-*N*-(3,5-dichlorophenyl)-*N*-methylbenzamide (72 mg, 0.19 mmol) and 5% Pd/C (wet Degussa-type E101, 12 mg, theor. 0.0029 mmol Pd) in EtOAc (2 mL) was shaken under a hydrogen atmosphere (60 psi, rt, 1 h). The resulting mixture was filtered (diatomaceous earth) and the filtrate was concentrated in vacuo to afford a light orange oil which after flash chromatography afforded **19** (44 mg, 0.15 mmol) as a colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25–7.24 (m, 2H), 7.03 (s, 2H), 6.97 (d, J = 8.5 Hz, 1H), 7.39 (d, J = 8.5 Hz, 1H), 6.54 (m, 1H), 3.46 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.5, 160.5, 147.0, 135.6, 133.5, 130.0, 127.2, 125.2, 125.2, 118.2, 115.2, 39.1; MS (+EI) m/z 298 (MH^+, 70), 296 (MH^+, 100).

4.2.3. 2-(*N*-(3,5-Dichlorophenyl)-2-hydroxybenzamido)acetic acid (20)

To a stirred solution of **10** (0.250 g, 0.672 mmol), prepared according to the method described in the preceding section, and sodium *tert*-butoxide (0.130 g, 1.34 mmol) in anhydrous 1,2-dimethoxyethane (7 mL) was added (rt, 5 min) neat *tert*-butylbromoacetate (0.22 mL, 1.3 mmol) and the resulting solution was stirred (rt, 90 min). After aqueous work-up (EtOAc, H_2O), the organic layer was dried ($MgSO_4$) and concentrated in vacuo and filtered through a plug of silica gel (hexanes/EtOAc, 5:1) to afford *tert*-butyl 2-(2-(benzyloxy)-*N*-(3,5-dichlorophenyl)benzamido)acetate as a crude colorless oil (0.285 g) which was used without further purification: MS (+EI) m/z 510 (MNa^+, 20), 508 (MNa^+, 30), 432 ((M-56)H^+, 70), 430 ((M-56)H^+, 100). A suspension of this intermediate oil (0.285 g, theor. 0.586 mmol) and 5% Pd/C (wet Degussa-type E101, 22 mg, theor. 0.0053 mmol Pd) in EtOAc (3 mL) was shaken under a hydrogen atmosphere (60 psi, rt, 3 h). The resulting mixture was filtered (diatomaceous earth) and the filtrate was concentrated in vacuo. The crystalline solid that was obtained was triturated (hexanes) to afford *tert*-butyl 2-(*N*-(3,5-dichlorophenyl)-2-hydroxybenzamido)acetate as a white crystalline solid (0.148 g, 0.373 mmol): ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.2 (br s, 1H), 7.27–7.24 (m with $CHCl_3$, ~2H), 7.09 (s, 2H), 6.96 (d, J = 8.0 Hz, 1H), 6.79 (d, J = 8.5 Hz, 1H), 6.55 (m, 1H), 4.41 (s, 2H), 1.50 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.3, 167.5, 160.1, 145.9, 135.5, 133.7, 129.9, 127.6, 125.6, 118.4, 118.2, 115.2, 82.9, 53.6, 28.0; MS (+EI) m/z 396 (MH^+, 70), 394 (MH^+, 100). A solution of *tert*-butyl 2-(*N*-(3,5-dichlorophenyl)-2-hydroxybenzamido)acetate (109 mg, 0.275 mmol) in CH_2Cl_2:TFA (1:1 v/v, 4 mL) was stirred (rt, 1 h) and then concentrated in vacuo. The resulting solid was crystallized (hexanes/EtOAc) to afford **20** as a white crystalline solid (50 mg, 0.15 mmol): ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.89 (br s, 1H), 9.87 (br s, 1H), 7.39 (m, 1H), 7.25 (s, 2H), 7.13 (m, 1H), 7.06 (d, J = 7.5 Hz, 1H), 6.74–6.71 (m, 2H), 4.51 (s, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.4, 168.6, 153.5, 145.0, 133.1, 130.9, 128.4, 126.1, 125.9, 123.3, 118.8, 115.6, 50.5; MS (+EI) m/z 340 (MH^+, 70), 338 (MH^+, 100).

4.2.4. 2-(3,5-Dichlorophenyl)-4-hydroxyisoindoline-1,3-dione (22)

A solution of 3-hydroxyphthalic anhydride (701 mg, 4.27 mmol) and 3,5-dichloroaniline (692 mg, 4.27 mmol) in glacial acetic acid (10 mL) was refluxed (2 h) and the resulting suspension was allowed to cool (rt). The precipitant was collected, washed (AcOH) and dried in vacuo to afford **22** as fine white needles (1.17 g, 3.80 mmol): ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.20 (s, 1H), 7.70–7.59 (m, 2H), 7.58 (d, J = 4.0 Hz, 2H), 7.39 (t, J = 4.0 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 166.1, 164.9, 155.8, 136.4, 134.4, 133.8, 133.2, 127.4, 126.2, 123.5, 114.5; MS (−EI) m/z 308 (M^-, 70), 306 (M^-, 100).

4.3. General bio-assay methods

All cell culture manipulations were performed in a biological safety cabinet, unless otherwise indicated. Media and additives were prepared and maintained under aseptic conditions with storage in the refrigerator up to one month.

4.4. Fluorescence immunoassay protocol

The cytokine detection assays used a stimulatory mixture consisting of *mu*CD40L, *mu*IL-18 and *mu*IFNγ to induce IL-12p40 and IL-6 secretion by a murine dendritic cell line, 'DC-B'.[20,21] DC-B cells were cultured in growth medium (i.e. recipe follows below) with 60 ml total volume in T-175 tissue culture flasks maintained in a 37 ± 2 °C, 5 ± 1% CO_2 high humidity incubator. Non-adherent cells were collected and diluted (1:3 v/v to 2.5×10^5/mL) every 48 h. DC-B cell growth medium was prepared by combining the following: Hepes buffer (1 M, 10 mL, pH 7.4); sodium pyruvate (10 mL, 100×, Gibco #11360-070); MEM Vitamin Solution (4 mL, 100×, Gibco #1120-0511); essential amino acids without glutamine (8 mL, 50×, Gibco #11130-051); non-essential amino acids (4 mL, 100×, Gibco #11140-050); PSG (10 mL); 2-ME (1 mL, 1000×, Gibco #21985-023); FBS (10%, heat inactivated); GM-CSF (20 ng/mL), and diluted with bicarbonate (to 1 L). Assay medium for DC-B cells was prepared by combining the following: in-house murine GM-CSF (20 ng/mL, 10 mL), non-essential amino acids (1% aqueous, 10 mL); sodium pyruvate (1% aqueous, 10 mL), PSG (1% aqueous, 10 mL), Hepes buffer (0.01 M, 10 mL), FBS (0.1% aqueous, heat inactivated), 50% aqueous Click's Reagent. Immunoassay reagents include: in-house murine GM-CSF (500 g/mL), murine IL-6 and IL-12p4. ELISA antibody pairs included purified rat anti-mouse IL-6 monoclonal antibody (PharMingen #554400), biotinylated rat anti-mouse IL-6 monoclonal antibody (PharMingen #554402), purified rat anti-mouse IL-12p40/p70 monoclonal antibody (PharMingen #551219), biotinylated rat anti-mouse IL-12p40/p70 monoclonal antibody (PharMingen #554476), DELFIA Assay Buffer™ (Wallac #1244-111), europium-labeled streptavidin, (Wallac #1244-360, 0.1 mg/mL) and Enhancement Solution™ (Wallac #1244-105). Capture antibodies (i.e. IL12-p40 or IL-6) were diluted (1:250 v/v) in PBS. The secondary capture antibody for IL-12p40 and IL-6 were diluted in heat inactivated FBS (10% in PBS, 1:250 and 1:500, v/v dilution, respectively). Assay plates were high-binding 384-well plates (Greiner #781077) and cell culture plates were black wall/clear bottom 384-well plates (Costar #3712).

To solutions of the test compounds (40 μM in DMSO/assay media, 0.4% v/v) in assay media (12.5 μL per well in 384-well, cell culture plates) were added DC-B cells in DELFIA Assay Buffer™ (27.5 μL/well, cell concentration of 7.27×10^5/mL) followed by the cytokine stimulation cocktail (10 μL/well, or assay media for control wells) to achieve the desired final test compound concentration and volume (i.e. 10 μM, 50 μL/well). Stimulation cocktail was prepared so that the final concentration after the dilution that was just described resulted in the desired concentration for

each cytokine: murine CD40L (2 ng/mL), murine IL-18 (40 ng/mL) and murine IFNγ (20 ng/mL). The resulting assay plates were incubated (18–24 h, 37 °C incubator with 5% CO_2) after which the supernatant (i.e. containing test compound or control) was transferred to 384-well assay plates that were pre-coated, at 4 °C, with capture antibody (i.e. 20 μL/well, either anti-IL-12p40 or anti-IL-6). These assay plates were then incubated (2 h, rt) and then washed with PBST (i.e. 0.05% Tween 20 in PBS). A biotinylated secondary antibody (20 μL/well) was added and the plates were incubated (1 h, rt). After washing with PBST, europium-labeled streptavidin (20 μL/well, diluted 1:1670 v/v in Delfia Assay Buffer™) was added and the plates were incubated (30 min, rt). Microtiter plates were analyzed (europium counts at 615 nm) using a fluorescence microplate reader (Victor^2, Wallac). Assay experimental data points were calculated as percentage of control (POC) and dose–response plots represent POC versus concentration. Each concentration in the dose–response plots was comprised of three assay experimental data points. The concentrations tested were over a range of ten values (i.e. 0.0005, 0.0015, 0.0046, 0.014, 0.041, 0.12, 0.37, 1.11, 3.33, and 10.0, all μM). IC50's, defined as the inhibition at 50% of response, were determined from these dose–response curves. Assay data analysis was performed using Spotfire Decision Site 8.1 (www.Tibco.com) and Activity Base 5.0 queried with SARgen (www.idbs.com).

4.5. Cytotoxicity and recombinant protein profiling assays

Cytotoxicity was assessed in our dendritic cell line by using the CellTiter-Glo™ cell viability protocol (www.promega.com). The dendritic cells, treated with test compound, were then stimulated according to the same protocol described in the preceding section for the IL-12p40 assay protocol (i.e. incubated 18 h with murine CD40L, murine IL-18, and murine IFNγ). The CellTiter-Glo™ reagent was then added in equal volume to culture volume and incubated (i.e. 2 min with orbital shaking to induce cell lysis followed by 10 min of incubation without shaking). Luminescence was then measured by fluorescence microplate reader. Cytotoxicity was not observed, for any compound reported here, below 100 nM. Compounds demonstrating selective inhibition of IL-12p40 production and including **3** and **9** were counter-screened against a panel of 75× receptor binding and 71× enzyme inhibition assays as well as routine, in-house biochemical assays and in no instance was significant activity observed that might indicate a target or class of targets (i.e. <30% inhibition at either 10 μM or 25 μM, depending upon the assay, as performed at Cerep, www.cerep.com). Experiments using the selective salicyanilide inhibitors of IL-12p40 production, described here, as probes for biochemical target identification are underway and will be reported elsewhere.

4.6. Calculated physical properties

The calculated physical properties such as pK_a were determined using ACD/LogD Suite™ 9.0 (ACD/Labs, Toronto, ON, Canada, www.acdlabs.com).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.07.024.

References and notes

1. Trinchieri, G.; Pflanz, S.; Kastelein, R. A. *Immunity* **2003**, *19*, 641–644.
2. Gately, M. K.; Renzetti, L. M.; Magram, J.; Stern, A. S.; Adorini, L.; Gubler, U.; Presky, D. H. *Annu. Rev. Immunol.* **1998**, *16*, 495–521.
3. Mannon, P. J.; Fuss, I. J.; Mayer, L.; Elson, C. O.; Sandborn, W. J.; Present, D.; Dolin, B.; Goodman, N.; Groden, C.; Hornung, R. L.; Quezado, M.; Neurath, M. F.; Salfeld, J.; Veldman, G. M.; Schwertschlag, U.; Strober, W. *New Engl. J. Med.* **2004**, *351*, 2069–2079.
4. Toichi, E.; Torres, G.; McCormick, T. S.; Chang, T.; Mascelli, M. A.; Kauffman, C. L.; Aria, N.; Gottlieb, A. B.; Everitt, D. E.; Frederick, B.; Pendley, C. E.; Cooper, K. D. *J. Immunol.* **2006**, *177*(7), 4917–4926.
5. Burakoff, R.; Barish, C. F.; Riff, D.; Pruitt, R.; Chey, W. Y.; Farraye, F. A.; Shafran, I.; Katz, S.; Krone, C. L.; Vander, V. M.; Stevens, C.; Sherman, M. L.; Jacobson, E.; Bleday, R. *Inflamm. Bowel Dis.* **2006**, *12*(7), 558–565.
6. Coopera, A. M.; Khader, S. A. *Trends Immunol.* **2007**, *28*(1), 33–38.
7. For leading references on anti-bacterial activities of salicylanilides see the following and Ref. 8 Waisser, K.; Matyk, J.; Divišová, H.; Husáková, P.; Kuneš, J.; Klimešová, V.; Kaustová, J.; Möllmann, U.; Dahse, H.-M.; Miko, M. *Arch. Pharm.* **2006**, *339*(11), 616–620.
8. Fuente, R. De La; Sonawane, N. D.; Arumainayagam, D.; Verkman, A. S. *Br. J. Pharmacol.* **2006**, *149*, 551–559.
9. Kasckow, J. W.; Tabira, T.; Itoyama, Y.; Kuroiwa, Y.; Takenaka, A.; Nagai, Y. *Jpn. J. Exp. Med.* **1981**, *515*, 293–298.
10. Zhu, X.-F.; Wang, J.-S.; Cai, L.-L.; Zeng, Y.-X.; Yang, D. *Cancer Sci.* **2006**, *971*, 84–89.
11. Agrawal, V. K.; Sharma, S. *Pharmazie* **1984**, *396*, 373–378.
12. Compounds **1** and **2** were not included among the original compounds that we evaluated for inhibition of IL-12p40 production.
13. Inhibition of IL-12p40 production was also assessed using primary human dendritic cells however the human donor-to-donor cell variability of these primary cells and the difficulty posed by their routine collection made their use difficult thus a murine dentritic cell line was used for routine SAR development.
14. A similar observation can be made by comparing compounds **13** and **8**. One hypothesis for this effect would invoke subtle differences in interactions with different target proteins.
15. Calculated pK_a's for **11** (6.6) and **9** (8.2) were determined using ACD/LogD Suite™ 9.0, Advanced Chemistry Development, Inc., (ACD/Labs), Toronto, ON, Canada, www.acdlabs.com.
16. Biochemical target identification, in order to understand the mechanism of action of these salicylanilides, will be reported elsewhere.
17. Thomas, M. G.; Allanson, N. M. Brit. UK Pat. Appl. 2002, 13 pp.
18. Liechti, C.; Sequin, U.; Bold, G.; Furet, P.; Meyer, T.; Traxler, P. *Eur. J. Med. Chem.* **2004**, *391*, 11–26.
19. Muto, S.; Nagano, T.; Saotome, T.; Itai, A. *PCT Int. Appl.* **2002**, 313.
20. Eriksson, U.; Kurrer, M. O.; Sonderegger, I.; Iezzi, G.; Tafuri, A.; Hunziker, L.; Suzuki, S.; Bachmaier, K.; Bingisser, R. M.; Penninger, J. M.; Kopf, M. *J. Exp. Med.* **2003**, *197*(3), 323–331.
21. Pasare, C.; Medzhitov, R. *Science* **2003**, *299*(5609), 1033–1036.