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An Approach to the Identification of Potent Inhibitors of Influenza Virus Fusion Using Parallel Synthesis Methodology

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Abstract—Structure–activity studies associated with the salicylic acid-derived inhibitor of influenza fusion, BMY-27709, were examined using a parallel synthesis approach. This SAR survey led to the discovery of potent influenza inhibitory activity in a series of aromatic amides and thioamides derived from 1,3,3-trimethyl-5-hydroxycyclohexylmethylamine. Select compounds were characterized as inhibitors of the H1 subtype of influenza A viruses that act by preventing the pH-induced fusion process, thereby blocking viral entry into host cells. In a plaque-reduction assay, the most potent inhibitors displayed EC_{50} values of 0.02–0.14 μg/mL.

Problematic winter outbreaks of influenza continue to emerge annually and are associated with significant morbidity and, in certain populations, mortality. These annual epidemics are driven by antigenic drift, a consequence of the poor fidelity of the influenza virus RNA polymerase, whilst the far more insidious pandemics that occur less frequently but generally impose a much greater disease burden, result from antigenic shift, the production of reassortant viruses.[1–4] In recent years, a strategy of prophylaxis that depends upon vaccination campaigns conducted just prior to the onset of the influenza season has been implemented. However, the success of this approach is highly dependent upon an ability to accurately predict the anticipated circulating virus strains several months in advance to produce an appropriately effective vaccine. The search for effective drugs that can be used either as prophylactic or therapeutic agents for the prevention and treatment of influenza[4] has culminated with the recent approvals in the United States of the neuraminidase inhibitors zanamavir[5] and oseltamivir.[6] Both compounds are effective inhibitors of type A and type B influenza strains and early indications are that the development of resistance will not be a serious problem with either agent, which contrasts with the clinical observations with the M2 channel blockers amantadine and rimantadine.[1–4]

BMY-27709 (**1**) was recently identified as an effective and potent inhibitor of the H1 and H2 subtypes of influenza A virus strains in cell culture using a plaque reduction assay executed in a broad screening mode (Table 1).[7] This compound was subsequently shown to interfere with virus infectivity by preventing the low pH-induced conformational rearrangement of hemagglutinin into its fusogenic state, thereby blocking virus and host cell membrane fusion, an obligate event for virus entry.[8] An initial survey of the SAR associated with the salicylic acid element of **1** indicated an essential role for the 2-hydroxyl and revealed limited tolerance for additional substituents, except at the 5-position where CH_3 and Cl, compounds **2a** and **2b**, respectively, were found to be optimal.[9,10]

As part of an overall effort directed towards further defining the fusion-inhibiting pharmacophore and identifying compounds with both increased potency and inhibition of H3 influenza subtypes, we sought to rapidly and broadly probe the SAR, associated with the

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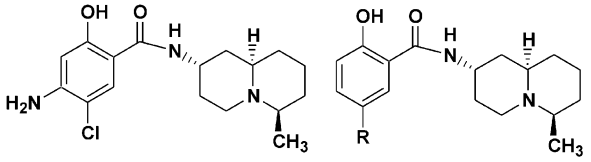
Table 1. Influenza inhibitory activity of individual compounds

Compd	Inhibition of hemolysis	Inhibition of influenza virus infectivity in cell culture	Cytotoxicity
	IC_{50} (μg/mL) + SD (*n*)	EC_{50} (μg/mL) + SD	CC_{50} (μg/mL)
1	0.44±0.21 (6)	2	>100
10a	0.15±0.049 (2)	0.136±0.047	50
10b	0.25 (1)	0.13±0.064	>100
11a	0.033±0.012 (6)	0.037±0.005	70
11b	0.025 (1)	0.03±0.015	80
13	2.5 (1)	Inactive	8–9
14	0.047±0.012 (3)	0.02	20

amine element.[11] The strategy adopted was dependent on parallel synthesis methodology with the objective being to identify structurally simpler and synthetically more accessible surrogates for the quinolizidine that would facilitate further SAR development.



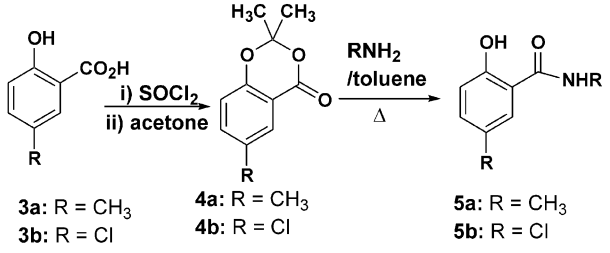
1 (BMY-27709)

2a: R = CH_3
2b: R = Cl

A series of salicylamide derivatives was synthesized by heating the cyclic acetonide **4**[11,12] with an amine in toluene, as summarized in Scheme 1.

A 3-fold excess of the amine was essential in order to drive this reaction to completion, necessitating that the product be filtered through an ion exchange (SAX) cartridge to remove excess amine.[13] Reactions were performed in parallel in batches of 20 and all reactions were evaluated by HPLC and MS. Products with correct mass that exhibited greater than 70% purity were submitted for biological evaluation without further purification, whilst those with the correct product but of lesser purity were purified by automated LC. The success rate was approximately 80%, with 175 products meeting the defined purity specifications from a total of 218 attempted reactions.

Amine **6** was identified from this library as a potent and effective surrogate for the quinolizidine moiety of **1** and **2**. Consequently, this amine (**6**) was used as the vehicle in a second library designed to complete the SAR survey by probing the effects of variation of the carboxylic acid

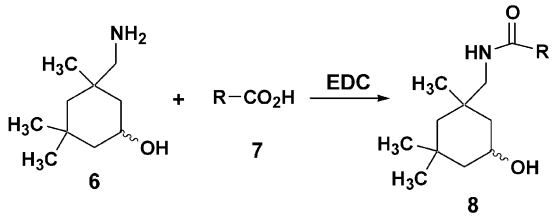


3a: R = CH_3
3b: R = Cl

4a: R = CH_3
4b: R = Cl

5a: R = CH_3
5b: R = Cl

Scheme 1.



6 **7** **8**

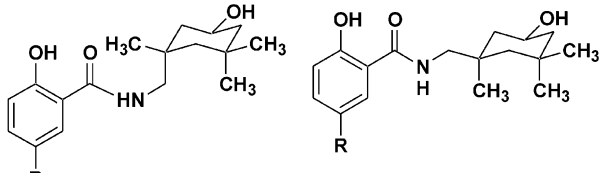
Scheme 2.

element. To this end, amine **6** was coupled with 200 structurally diverse carboxylic acids, using EDC as the activating agent, as depicted in Scheme 2.

The products were separated from excess carboxylic acid and the urea by-product by filtration through cartridges containing a mixture of SAX and SCX resins.[14] The success of the individual reactions was determined by HPLC and MS analysis using criteria identical to that established for the first library. The success rate was again 80%, with 160 compounds of sufficient purity to qualify for biological evaluation.

For both the libraries, diverse reagents were selected from the ACD collection using two dimensional atom-pair-based algorithm. From the initial library, **6** was identified as the only amine that provided potent and effective influenza inhibitors. The mixture of amide diastereomers **10a/11a** and the analogous chloro derivatives **10b/11b** both exhibited EC_{50}'s of 0.08 μg/mL for inhibition of influenza infectivity in tissue cell culture.

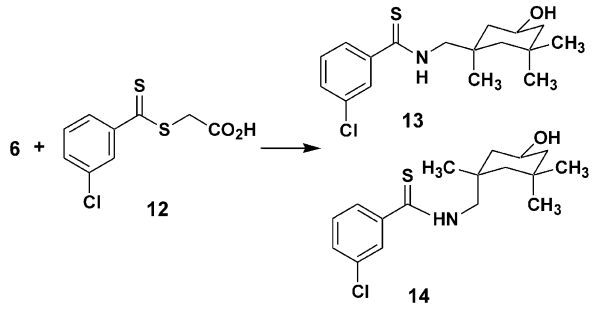
The individual diastereomers were readily resolved by chromatography[15] and their structures established from an analysis of NMR spectral data, with NOE experiments particularly informative. From the mixture of **10a** and **11a**, the chromatographically more mobile diastereomer was identified as the axial amide **10a** based on the observation of enhancement of both diastereotopic NCH_2 protons at δ 3.11 and 3.74, the axial CH_3 and the flanking equatorial protons when the C*H*OH proton was irradiated.[16] In contrast, irradiation of the C*H*OH proton of the more polar material, **11a**, resulted in enhancement only of the flanking equatorial protons and the two CH_3 groups at δ 1.00 and 1.04.[17] No enhancement of the NCH_2 protons, a multiplet at δ 3.20, was observed. The structures of **10a**, **10b**, **11a**, and **11b** were confirmed by single crystal X-ray crystallography.[18]



10a: R = Me
10b: R = Cl

11a: R = Me
11b: R = Cl

The library of amides prepared from **6** produced only a single active member, characterized initially as a mixture of diastereomers, derived from the carboxylic acid **12**. However, the active compound was identified as a



Scheme 3.

thioamide rather than an amide, due to the alternative reaction manifold summarized in Scheme 3.

The diastereomers **13** and **14** were separated by chromatography and their relative stereochemistry established after examination of ^{1}H NMR spectral data.[19] The chromatographically more mobile isomer was identified as the equatorial thioamide **13**[19] whilst in the more polar diastereomer, **14**, the thioamide moiety is axially disposed.[20] Diagnostic were the NCH_2 protons which are diastereotopic in the axial isomer **14** and resonate as individual signals at δ 3.54 and 4.29. In contrast, the NCH_2 protons of the equatorial isomer **13** are overlapping doublets of doublets at δ 3.69 and 3.73. The assignment of the relative stereochemistry of **14** was confirmed by single crystal X-ray analysis.[21]

The more potent influenza inhibitor in this series proved to be the axial thioamide **14**, $EC_{50} = 0.02$ μg/mL, whilst the equatorial isomer **13** was inactive at concentrations below the half-maximally cytotoxic concentration (CC_{50}) of 10 μg/mL.

The influenza inhibitory activity of the amides **10** and **11** and the thioamides **13** and **14** provide additional insight into the structural elements required for this class of fusion inhibitor to fully express antiviral activity. That these compounds are fusion inhibitors was confirmed by evaluation in a hemolysis assay,[7,8,10] where the fusion event is more explicitly evaluated, and the results are presented in Table 1.

The prototypical influenza fusion inhibitor BMY-27709 (**1**) has been postulated to bind to a pocket on HA close to the amino terminus of the fusion peptide. In this model, the salicylamde functions as a carboxylic acid isostere,[22] engaging with the guanidine moiety of Arg106 via two complementary hydrogen bonds, the aromatic ring interacts with Phe110 and the methyl substituent of the quinolizidine ring interacts with Leu2 of the fusion peptide.[8,10] The amides **10** and **11** retain these key functional elements and can readily establish the same interactions with the HA protein. Under these circumstances, the potency differences between the axial (**10**) and equatorial (**11**) isomers reflect the extent of complementarity with the HA protein. In contrast, the thioamides isomers **13** and **14** demonstrate marked differences in potency with the equatorial isomer **13** inactive in the cell culture assay although weak activity was detected in the hemolysis assay. The thioamide moiety presumably establishes a different mode of binding to Arg106 such that only the axial isomer **14** is capable of establishing the full complement of molecular interactions required to interfere with the rearrangement of HA. These compounds are notable by the absence of the basic amine element found in **1** and the structurally simpler but related piperidine-based influenza fusion inhibitor,[23] both of which have been postulated to form a charge-charge interaction with Glu105 of HA2.[8,23] Following completion of this work, scientists at Lilly described the discovery of a series of ureas derived from amine **6** that also exhibit influenza inhibitory activity.[24] Interestingly, in this series the axial and equatorial isomers exhibited equipotent influenza inhibitory activity as did the individual enantiomers.

In summary, we have described a strategic approach that has very effectively identified highly potent inhibitors of H1 influenza fusion based on the lead BMY-27709 (**1**). However, the marked increase in potency against H1 influenza was not accompanied by significant H3 influenza inhibitory activity (data not shown), although the cyclohexanol template identified in this study provided a synthetically more accessible and structurally more versatile vehicle for SAR elaboration.[25]

Acknowledgements

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13. SAX cartridges (6 mL/1 g) were pretreated with 10 mL 0.5 M KOH/EtOH and rinsed with 20 mL of CH_2Cl_2. The crude reaction mixture (0.25 mmol) was loaded on the cartridge and washed with 50 mL of CH_2Cl_2 to remove excess amine. The desired salicylamide was finally eluted with 10 mL of CH_2Cl_2. Purifications were automated using a Zymark Benchmate™ instrument.
14. The crude reaction mixture in CH_2Cl_2 was loaded on to a 6 mL cartridge containing 0.5 g of SAX and 0.5 g of SCX resin. The ion exchange cartridge was then washed with 10 mL of CH_2Cl_2 to obtain the desired product. Purifications were automated using a Zymark Benchmate™ instrument.
15. The mixtures of diastereomeric amides **10** and **11** were separated by chromatographing over silica gel using 50% EtOAc/hexane as the mobile phase.
16. ^{1}H NMR spectral data for **10a** ($CDCl_3$) δ 0.96 (3H, s, equatorial CH_3), 0.97 (3H, s, axial CH_3), 1.02 (3H, s, equatorial CH_3), 0.95–1.10 (4H, m), 1.34 (1H, d, $J=14$ Hz), 1.72 (1H, d, $J=14$ Hz), 1.82 (1H, d, $J=14$ Hz), 2.25 (3H, s, Ar–CH_3), 3.11 (1H, dd, $J=13$ Hz, $J'=5$ Hz, NCH_2), 3.74 (1H, dd, $J=13$ Hz, $J'=5$ Hz, NCH_2), 4.02 (1H, m, C*H*OH), 6.67 (1H, m, NH), 6.84 (1H, d, $J=9$ Hz), 7.16 (2H, m), 11.97 (1H, s, O*H*).
17. ^{1}H NMR spectral data for **11a** ($CDCl_3$) δ 0.94 (3H, s, equatorial CH_3), 1.00 (3H, s, axial CH_3), 1.04 (3H, s, axial CH_3), 0.99–1.06 (3H, m), 1.72 (2H, m), 1.88 (1H, bs), 2.26 (3H, s, Ar–CH_3), 3.20 (2H, m, NCH_2), 3.94 (1H, m, C*H*OH), 6.55 (1H, m, NH), 6.85 (1H, d, $J=8$ Hz), 7.15 (2H, m), 11.97 (1H, s, O*H*).
18. Single crystal X-ray diffraction data were collected at room temperature using a Nonius CAD-4 diffractometer equipped with Cu K_α radiation. Crystal data for **10a**: Crystallized from EtOAc; $C_{18}H_{27}NO_3$; space group *P1-bar*; $a=7.6211(5)$ Å; $b=10.6651(8)$ Å; $c=11.6580(8)$ Å, $\alpha=112.59(1)°$, $\beta=92.50(1)°$, $\gamma=102.60(1)°$, $V=845.2(1)$ Å^3; $Z=2$; $d_x=1.204$ g cm^{-3}; data collected at 295 K; 3687 reflections measured, 2669 observed ($I\geq3\sigma$); 200 parameters refined; $R(F)=0.050$; $wR(F)=0.047$; $S=2.787$. Crystal data for **11a**: Crystallized from CH_3CN; $C_{18}H_{27}NO_3$; space group *P1-bar*; $a=7.622(2)$ Å; $b=10.292(2)$ Å; $c=12.342(2)$ Å; $\alpha=68.03(1)°$, $\beta=74.11(1)°$; $\gamma=75.53(1)°$; $V=851.8(3)$ Å^3; $Z=2$; $d_x=1.191$ g cm^{-3}; data collected at 295 K; 3770 reflections measured, 2416 observed ($I>3\sigma$); 200 parameters refined; $R(F)=0.09$; $wR(F)=0.11$; $S=4.26$. The coordinates for **10a**, **10b**, **11a**, and **11b** have been deposited in the Cambridge Crystallographic Database (deposition nos: CCDC163011–CCDC163014).
19. ^{1}H NMR spectral data for **13** ($CDCl_3$) δ 1.01 (3H, s), 1.08 (3H, s), 1.19 (3H, s), 1.05–1.40 (5H, m), 1.75–1.88 (2H, m), 3.69 (1H, dd, $J=13.7$ Hz, $J'=6$ Hz, one NCH_2), 3.73 (1H, dd, $J=13.7$ Hz, $J'=6$ Hz, one NCH_2), 4.01 (1H, m, C*H*OH), 7.34 (1H, t, $J=8$ Hz), 7.44 (1H, d, $J=8$ Hz), 7.55 (1H, bs), 7.57 (1H, d, $J=8$ Hz), 7.71 (1H, s).
20. ^{1}H NMR spectral data for **14** ($CDCl_3$) δ 1.01 (3H, s), 1.09 (6H, s), 0.93–1.20 (4H, m), 1.43 (1H, m), 1.81 (1H, dd, $J=12.4$ Hz, $J'=2$ Hz), 1.92 (1H, dd, $J=13.1$ Hz, $J'=2$ Hz), 3.54 (1H, dd, $J=14.2$ Hz, $J'=4.7$ Hz, one NCH_2), 4.16 (1H, m, C*H*OH), 4.29 (1H, dd, $J=14.2$ Hz, $J'=6.8$ Hz, one NCH_2), 7.35 (1H, t, $J=8$ Hz), 7.45 (1H, d, $J=8$ Hz), 7.48 (1H, bs), 7.57 (1H, dd, $J=8$ Hz, $J'=0.7$ Hz), 7.71 (1H, d, $J=1.7$ Hz).
21. Crystal data for **14**: Crystallized from EtOAc; $C_{17}H_{24}NOSCl$; space group $P2_1/n$; $a=11.6378(6)$ Å; $b=110.9443(6)$ Å; $c=25.296(1)$ Å; $\beta=94.857(5)°$; $V=3503.6(3)$ Å^3; $Z=8$; $d_x=1.186$ g cm^{-3}; data collected at 295 K; 7556 reflections measured, 5545 observed ($I>3\sigma$); 380 parameters refined; $R(F)=0.048$; $wR(F)=0.058$; $S=1.93$. The coordinates for **14** have been deposited in the Cambridge Crystallographic Database (deposition no. CCDC163015).
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