Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 24, pp. 2931-2936, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00542-2

NOVEL INHIBITORS OF INFLUENZA SIALIDASES RELATED TO GG167
Structure-Activity, Crystallographic and Molecular Dynamics Studies with 4H-Pyran-2-Carboxylic Acid 6-Carboxamides

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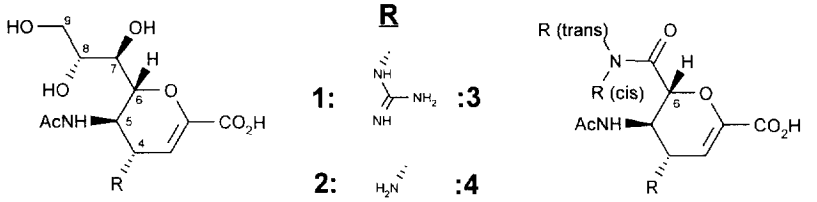
Abstract: The structure-activity relationships of a series of 4-amino and guanidino-4H-pyran-2-carboxylic acid 6-carboxamides are described. These compounds represent a new class of inhibitor of influenza sialidases and are particularly active against influenza A sialidase. The binding of the N-phenethyl-N-propylamide **4l** to influenza A and B sialidases has been investigated using X-ray crystallography and molecular dynamics simulations. Our results suggest that formation of a hitherto unobserved intramolecular salt bridge within the enzymes may account for the observed activity and selectivity of the series.

Introduction

Inhibitors of influenza virus sialidases have potential for the prophylaxis and treatment of influenza infections.[1] GG167 **1** is the most potent reported inhibitor of both influenza A and B virus sialidases and is currently undergoing clinical evaluation.[2] X-ray studies with these sialidases have shown that the 8- and 9-hydroxyl groups of sialic acid and inhibitors such as GG167 make hydrogen bonding interactions with the active sites,[3] and SAR studies based upon GG167 have confirmed the importance of the glycerol sidechain for enzyme affinity.[4] However, recently we reported that it is possible to replace the polar 6-glycerol group in **1** and **2** with a more lipophilic propylamide sidechain and achieve even better inhibitory activity against influenza A sialidase.[5] Herein we describe further 6-carboxamide analogues which were prepared in order to explore the structural requirements of the potent but selective inhibition of the influenza A enzyme. In addition, we have determined the binding of a representative carboxamide **4l** (R_{trans} = $PhCH_2CH_2$, R_{cis} = Propyl) by X-ray crystallography and applied molecular dynamics simulations to provide an explanation for the observed activity and selectivity of the series.



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Structure-Activity Studies

Synthesis and biological evaluation of compounds were carried out as described previously. Rapid initial exploration of SAR in the new series was achieved through the preparation of a chemical library.[6] Data for representative amides is summarised in the table below:[5,7]

			Sialidase IC_{50} (μM)[8]				
			4-Guanidino			4-Amino	
R_{trans}*	R_{cis}		Flu A	Flu B		Flu A	Flu B
$-CH_3$	-H	**3a**	7	-	**4a**	190	80
$-CH_2CH_3$	-H				**4b**	13	41
$-(CH_2)_2CH_3$	-H	**3c**	0.5	4.4	**4c**	19	50
$-(CH_2)_2Ph$	-H				**4d**	12	67
$-CH_3$	$-CH_3$	**3e**	0.025	11	**4e**	2.4	61
$-(CH_2)_2CH_3$	$-CH_3$	**3f**	0.004	4.5	**4f**	0.18	23
$-(CH_2)_2Ph$	$-CH_3$				**4g**	0.32	51
$-CH_2CH_3$	$-CH_2CH_3$	**3h**	0.001	0.5	**4h**	0.003	0.4
$-(CH_2)_2CH_3$	$-CH_2CH_3$				**4i**	0.003	0.4
$-(CH_2)_2Ph$	$-CH_2CH_3$				**4j**	0.005	8
$-(CH_2)_2CH_3$	$-(CH_2)_2CH_3$	**3k**	0.002	0.5	**4k**	0.003	2
$-(CH_2)_2Ph$	$-(CH_2)_2CH_3$	**3l**	0.005	0.8	**4l**	0.005	2
$-(CH_2)_2$– 	$-(CH_2)_2CH_3$				**4m**	0.003	2.3
$-(CH_2)_8CH_3$	$-(CH_2)_2CH_3$				**4n**	0.023	37
$-(CH_2)_3CH_3$	$-(CH_2)_2CH_3$				**4o**	0.004	1
$-(CH_2)_3CH_3$	$-(CH_2)_3CH_3$	**3p**	0.016	31	**4p**	0.45	>430
Glycerol		GG167 **1**	0.005	0.004	**2**	0.3	0.4

(* The larger of the two amide substituents is assumed to occupy predominantly the R_{trans} configuration.)

The secondary carboxamides **4a-d** show similar weak inhibition of both influenza A and B sialidases. However, introduction of an additional alkyl substituent (R_{cis}) results in a pronounced improvement in activity against the A enzyme but has relatively little effect on activity against the B enzyme. The tertiary amides **4e-p** thus show marked selectivity (30 to 1000-fold) for influenza A sialidase. Excellent activity against the A enzyme is attainable with a variety of different sized R_{trans} substituents larger than methyl, but the data suggests a more restricted requirement for the R_{cis} group. Thus, optimum activity is achieved

when R_{cis} is an ethyl or *n*-propyl group (**4h-o**), with the smaller methyl (**4f-g**) and larger *n*-butyl group (**4p**) both showing reduced activity.

4-Guanidino analogues **3** are more active than corresponding 4-amino compounds **4** but the improvement is generally less than observed with the 6-glycerol series (**1** and **2**), especially where the 4-amino analogue is already highly active (**4h,k-l**).

X-Ray Crystallographic Studies

Protein crystallography with representative influenza A (N9) and B (Beijing) sialidases has been used to compare the binding of GG167 and the N-phenethyl-N-propylamide **4l** and to rationalise the observed structure-activity relationships.

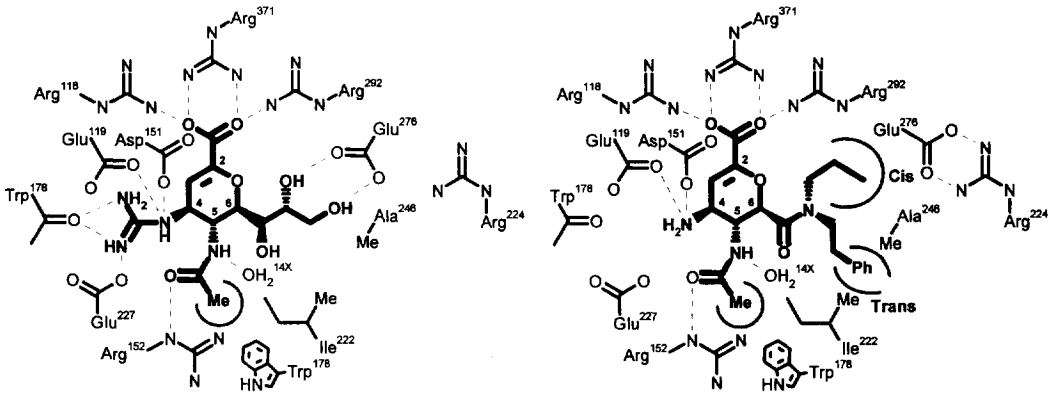

Figure 1. Schematic representation of binding of GG167 **1** and N-phenethyl-N-propylamide **4l** to the influenza sialidase active site.[9]

Carboxamide versus GG167 Binding

The dihydropyran portion of the carboxamide **4l** binds to both the A and B enzymes in essentially the same manner as that observed for GG167 **1** (Figure 1).[3] Thus, the 1-carboxylic acid interacts with Arg^{118}, Arg^{292} and Arg^{371}; the 4-amino group produces favourable electrostatic interactions with Glu^{119} and Asp^{151}; and the 5-acetamido group occupies a hydrophobic pocket (Trp^{178} and Ile^{222}) in addition to forming hydrogen bonds with Arg^{152} and a crystallographically bound water molecule (14X).[9] However, a significant difference from GG167 and other previously determined structures is observed in the region into which the carboxamide sidechain binds, as shown schematically in Figure 1 and in the crystallographic representations of Figures 2 and 3. Whereas in previous structures the glycerol moiety (or, in the case of native enzyme, solvent water) forms intermolecular hydrogen bonds with Glu^{276}, these

interactions are no longer available for the carboxamide **4l**. Instead, the Glu^{276} sidechain adopts an entirely different conformation, in which the carboxylate group forms an intramolecular salt bridge with the guanidino sidechain of Arg^{224} (the position of the latter is not significantly altered from previous structures). The result of this salt bridge formation is that a previously unrecognised lipophilic pocket becomes available for the R_{cis} *n*-propyl substituent to occupy. Furthermore, the size of this new pocket appears optimal for an ethyl or propyl group, in accord with the structure-activity relationships derived from Table 1. The R_{trans} phenethyl substituent lies in an extended lipophilic cleft on the enzyme surface formed between Ile^{222} and Ala^{246} (Figure 3). Inspection of this region shows that the cleft can satisfactorily accommodate a variety of R_{trans} substituents; a picture also consistent with the observed structure-activity relationships.

Carboxamide-A Enzyme Complex versus Carboxamide-B Enzyme Complex

Comparison of the X-ray crystal structures of native influenza A and B sialidases shows, in general, a very high correspondence between backbone position and side chain torsion angles of the conserved active site residues. There are, however, some significant differences in the region binding the glycerol sidechain of GG167 and particularly in the position and sidechain conformation of Glu^{276}. Despite these differences, GG167 is able to bind to the active site of both the A and B enzymes with little or no distortion of their native structures, forming very similar hydrogen bonds between the 8- and 9- hydroxyl groups and the Glu^{276} carboxylates (Figure 2a and 2b, thin lines). Figure 2a shows that the formation of the salt bridge between Glu^{276} and Arg^{224} which occurs upon binding of the carboxamide **4l** to the A enzyme (thick lines) is accomplished by changes in the torsion angles of the Glu^{276} sidechain, and that this requires little or no distortion of the protein backbone. When compound **4l** binds to influenza B sialidase a similar salt bridge is observed. However, the small difference in the position of Glu^{276} in influenza B sialidase means that a significant distortion of the protein backbone has to occur in order to form the salt bridge (Figure 2b compare thick and thin lines). Distortions in the protein structure of influenza B sialidase also arise around the R_{trans} phenethyl substituent - high temperature factors are observed in the loop that forms one side of the lipophilic cleft (the loop containing Ala^{246}). Average temperature factors are observed in the complex with influenza A sialidase. It thus appears that the structural changes which accompany the binding of carboxamides such as **4l** are energetically less favourable in the B enzyme and this accounts for the observed specificity of the carboxamides for influenza A sialidase.

Molecular Modelling

Molecular dynamics simulations have been used to examine the binding of compounds **4d,g,j** and **l** to influenza A and B sialidases.[10] Salt bridges between Glu^{276} and Arg^{224}, were predicted in a significant number of the low energy structures which were generated for complexes involving tertiary amides **4g, j** and **l** and the A enzyme. However, in the corresponding simulations with influenza B sialidase, salt

bridges were generated very infrequently, in agreement with the notion that such conformations are energetically less favourable for the influenza B enzyme.

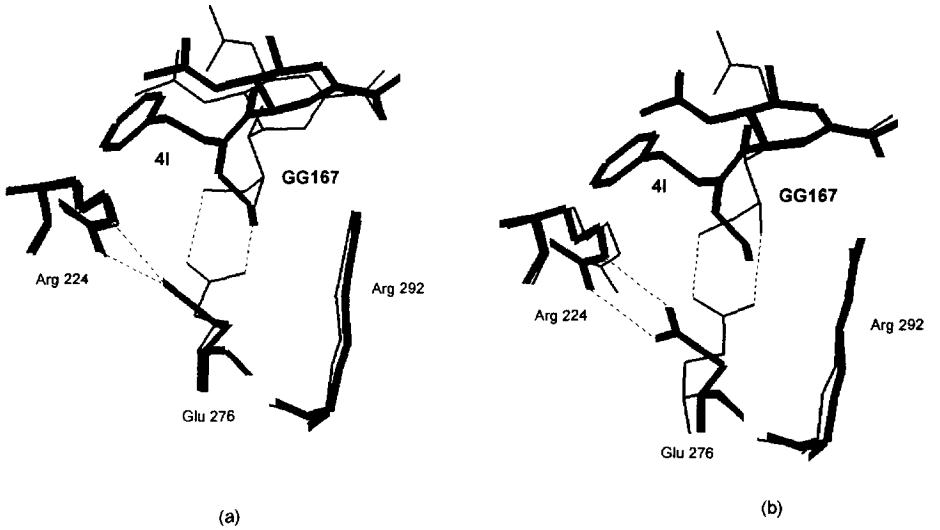

Figure 2. A comparison of the enzyme-ligand complexes of GG167 **1** (thin lines) and **4I** (thick lines) in (a) influenza A (N9) sialidase and (b) influenza B (Beijing) sialidase. Only those amino acid residues that define the *cis* pocket are shown (see text). The figure highlights that in influenza A sialidase the Glu^{276} Cα positions are similar in both complexes, whereas in influenza B there is a clear difference.

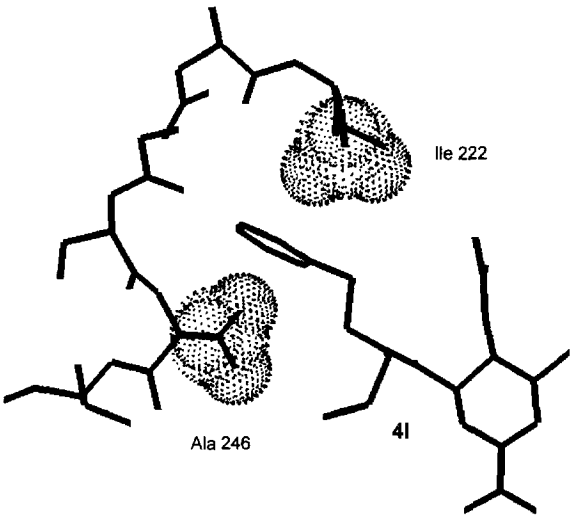

Figure 3. The phenyl ring of **4I** fits into the lipophilic *trans* pocket defined by Ala^{246} and Ile^{222} (shown here in influenza A sialidase). This mode of binding is very similar in both A and B sialidases.

Conclusion

Extremely potent but selective inhibitors of influenza A sialidase result when the glycerol sidechain of GG167 is replaced by a carboxamide. The R_{cis} amide substituent appears to be responsible for the high activity and selectivity of the tertiary carboxamides. The ease of intramolecular salt bridge formation between the sidechains of Glu^{276} and Arg^{224} may account for the observed selectivity between the A and B enzymes.

Acknowledgements: We wish to thank Safia Madar, Amanda Jowett and Fiona Record for their contributions to this work.

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6. A library containing 80 amides was prepared which included compound **4k**. Further details of this work will be reported in a full paper.
7. Woods, J.M.; Bethell, R.C.; Coates, J.A.V.; Healy, N.; Hiscox, S.A.; Pearson, B.A.; Ryan, M.; Ticehurst, J.; Tilling, J.; Walcott, S.M.; Penn, C.R. *Antimicrobial Agents Chemother.* **1993**, *37*, 1473
8. The IC_{50} values are calculated from the percent inhibition of enzyme activity in the presence of inhibitor relative to a positive (no inhibitor) control. All reactions were carried out in duplicate, and the mean values of these replicates used in the analysis of data. IC_{50}'s from different experiments differed by a factor of no more than 2.
9. Throughout the text the amino acid residues are numbered for influenza A sialidase
10. Molecular dynamics simulations were performed using a molecular dynamics/energy minimisation conformational searching protocol which incorporates multiple randomisation steps (Taylor, N.R.: von Itzstein, M. *J. Med. Chem.* **1994**, *37*, 616). The CVFF forcefield within the software package Discover was used (Biosym Technologies Inc., San Diego, USA). Fifteen energy minimised conformations were generated for each complex, and each simulation involved 70 ps of constrained molecular dynamics at 350 deg. K.

(*Received in Belgium* 13 *August* 1996; *accepted* 6 *November* 1996)