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New Approaches for the Inhibition of Influenza Neuraminidase

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The active site of influenza neuraminidases are highly conserved and are perceived to be very polar because of the large proportion of charged amino acid residues lining the active site. A key factor in the recent discovery and development of the orally active neuraminidase inhibitors oseltamivir and RWJ-270201 has been the successful exploitation of hydrophobic inhibitor-protein interactions in the "glycerol binding" subsite. Our investigation of a series of five member ring inhibitors of influenza neuraminidase has revealed another significant and previously unrecognized hydrophobic interaction for the putative "amine binding" subsite of neuraminidase. The optimization process for this site historically has targeted the flanking residues Glu 119 and Glu 227 (N2) for hydrogen bonding and electrostatic interactions. Using an iterative structure-based approach, we have uncovered a preference for hydrophobic substituents containing two contiguous sp^2 centers. The rationale for this preference correlates well with the conformational bias of these substituents and consequently with their ability to make a novel interaction with the hydrophobic face of these two glutamates. The SAR developed is consistent for both influenza A and B. We will describe an inhibitor series in which substitution for the precedented primary amine group with an optimized hydrophobic group resulted in approximately 200-fold potency improvements as measured by Ki.

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Pitfalls in the Biochemical Evaluation of Influenza Neuraminidase Inhibitors

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It is generally believed that the catalytic "head" domain of influenza neuraminidase possesses similar properties as the membrane associated neuraminidase present on the viral surface. We tested this assumption by measuring the Ki values for a set of compounds using purified neuraminidase catalytic heads as well as whole virus from A/N2, A/N9 and B strains of influenza virus. A heads-to-virus comparison using neuraminidase from A/N2/Tokyo/3/67 showed that the catalytic heads overestimated the inhibitory activity of many compounds by factors as high as 75-fold when compared to the Ki values measured with virus-associated neuraminidase. No significant heads/virus Ki value differences were observed with B/Memphis/3/89 or A/N9/NWS/G70c derived neuraminidase pairs. The virus-associated N2/Tokyo neuraminidase Ki values for some compounds often differed significantly from the values obtained using A/N9/G70c neuraminidase heads. This observation suggests that N9 crystal structure complexes may not be indicative of the binding orientations which occur when compounds are bound to N2 virus-associated neuraminidase, particularly in the Glu 278 region of the active site. We also observed that some compounds lose 3-5 fold in inhibitory activity, whereas others gain several fold, when the pH of the enzymatic assay is changed from pH 6.0 to 7.5. Thus, using N2 heads and a pH of 6.0 can overestimate the Ki values for some compounds by factors as high as 300-fold.

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Oseltamivir treatment of experimental influenza A/Texas/36/91 (H1N1) virus infection in humans: selection of a novel neuraminidase variant

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Early oral oseltamivir treatment of otherwise healthy volunteers experimentally infected with influenza A/Texas/36/91 (H1N1) virus is associated with significant antiviral clinical effects. Compared to placebo, oseltamivir reduced the median duration of viral shedding by 2 days and nasal titers by 100-fold at 24 h and 1000-fold at 36 h. Several oseltamivir-recipients experienced bimodal patterns of virus recovery from nasal lavages. Genotypic analysis identified 2 clinical isolates with a H274Y mutation in the neuraminidase (NA). This mutation resulted in the NA being ~ 400 fold less sensitive to inhibition by GS4071 (the active metabolite of oseltamivir). Importantly, the patients carrying the mutant virus showed no clinical deterioration and their symptom scores were typical of the treatment group. Following plaque purification, the mutant and the wild-type virus were compared for replicative ability *in vitro* and *in vivo*. Growth of the mutant in MDCK cells was reduced by at least 100 fold compared with wild-type. Infectivity of the mutant in mice was reduced by at least 1000 fold and in ferrets both infectivity and virus pathogenicity were significantly compromised. Of note, this mutation can also be selected by passage of A/Texas/36/91 *in vitro* in the presence of GS4071, but has not been seen in any naturally acquired influenza virus in patients treated with oseltamivir. In conclusion, a NA mutant of influenza A (H1N1) virus could be selected in a small number of experimentally infected subjects, although variants containing a H274Y mutation exhibit decreased infectivity in animal models. These findings indicate that the H274Y variant is biologically impaired and unlikely to limit the clinical usefulness of oseltamivir.

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Virological Assessment *in vitro* and *in vivo* of an Influenza H1N1 Virus with a H274Y Mutation in the Neuraminidase Gene

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A novel mutant of influenza virus A/Texas/36/91, carrying a neuraminidase with tyrosine in place of histidine at position 274 has been selected both *in vivo* by oseltamivir treatment, and *in vitro* by GS4071, the active metabolite of oseltamivir. The H274Y mutation effected reduced sensitivity of both neuraminidase (400-fold) and virus (3000-fold) to inhibition by GS4071 *in vitro*. The replicative ability of H274Y mutant virus in MDCK cells was reduced by 2 logs compared to wild-type virus. The infectivity and/or replicative ability of mutant virus in a mouse model of influenza infection was reduced by at least 3 logs. In the ferret model of influenza infection, infectivity of mutant virus was reduced by 2 logs and pathogenicity was significantly compromised. The H274Y mutation in the neuraminidase gene of influenza A/Texas/36/91 was stable through 6 days of replication *in vivo* in ferrets. Overall, the marked reduction in mutant virus replicative ability, infectivity and pathogenicity suggests that virus carrying H274Y in the neuraminidase gene is unlikely to be transmitted in man.