Recent Advances in Sialidase Inhibitors for the Treatment of Influenza

Paul W. Smith, *Glaxo Wellcome* Research and Development, Stevenage, Hertfordshire, UK

Influenza is one of the best-known and most-common diseases, but an effective therapy has still to be found. Therapeutic drugs have included both *Amantadine* and *Rimantidine*, which are active only against influenza A virus (they act by blocking the ion-channel function of the virus protein M2, which is not found in influenza B virus). Both drugs are characterized by side-effects at therapeutic doses and rapid development of resistance during treatment. Vaccines, on the other side, as a preventive approach, provide only a temporary solution, because viruses rapidly change their surface antigens.

The discovery of two influenza-virus surface proteins, haemagglutinin and sialidase, provided new targets. The haemagglutinin receptor recognizes host-cell adhesion molecules and thus helps virus penetration. Sialidase is an α-glycohydrolase which cleaves α-ketosidically-linked sialic acids from glycoproteins, glycolipids, and oligosaccarides and helps the spread of newly synthesized virions from infected cells and their movements within the respiratory tract.

Based on the transition state of sialic acid in the active site during the sialidase-catalyzed hydrolysis, the unsaturated sialic acid analogue Neu5Ac2en (DANA) and some derivatives have been synthesized. These early inhibitors (transition-state analogues) needed to be improved due to their disadvantages (non-selectivity, virus aggregation at host-cell surface, virus-spread inhibition in cell culture, no efficacy in animal models. and rapid renal clearance).

The X-ray crystal structure of sialic acid with influenza virus sialidase and GRID calculations made it possible to rationally design 4-guanidino-Neu5Ac2en (*Zanamivir*, GG167). The 4-guanidino group of *Zanamivir* increases the overall binding of the molecule by forming a salt bridge with the side-chain carboxy group of Glu 119 in the active site. This compound (which is at present in phase-III clinical trials) inhibits both influenza A and B virus sialidases and is selective for viral *vs.* bacterial and mammalian enzymes. It is effective when intranasally administered, but its bioavailability is very low when systemically administered. Structure-activity relationship (SAR) analysis of *Zanamivir* showed that removal of each of the four groups linked to the dihydropyran ring resulted in a dramatic loss of activity. Moving from the initial notion that the glycerol pocket could accomodate novel substituents and that the replacement of the glycerol side chain would result in more straightforward structures, 4-guanidino- and 4-amino-4*H*-pyran-6-carboxamides have been synthesized. SARs of this new set of compounds have revealed that amides with lipophilic sidechains are the most active, whereas secondary amides are weak inhibitors of both influenza A and B viral sialidases. In contrast, tertiary amides, which contain one or more small alkyl groups, are much more active. In particular, *cis*-amides are selective towards the influenza A virus enzyme. Crystallographic and molecular dynamic studies performed on a number of protein-ligand complexes showed that tertiary amides bind to both enzymes forming a planar salt bridge between the side chains of Glu276 and Arg224. Sialidase of influenza B has to undergo a conformational change in order to accommodate the *cis* substituent of the carboxamide. This energetically unfavourable distortion of the protein was suggested to cause selectivity of *cis*-amides of influenza A virus enzyme.

In order to further increase the selectivity towards influenza A virus sialidase, compound GS4071, a cyclohexyl analogue of sialic acid bearing an ether group instead of a carboxamide, and its ethyl ester prodrug GS4104 have been modified. Synthesis of 6-ether (acetal), 6-ketone and reverse-pyrane analogues has been performed in order to investigate the effects of cyclohexyl and pyran rings. Biological studies showed no increase in selectivity of new analogues, but rather a marked reduction in the activity of ethers and reversed-pyrans. The fact that ethers adopt the B-conformer in solution and that the aminomethyl group in reverse pyrans does not fit the basic pocket were suggested as explanation for the reduced activity. Based on these results, it was concluded that incorporation of a more flexible side chain into pyrans did not improve fluA/B selectivity and that subtle differences in pyran/cyclohexene ring geometry enabled GS4104 to achieve good binding *vs.* both fluA and -B sialidases.

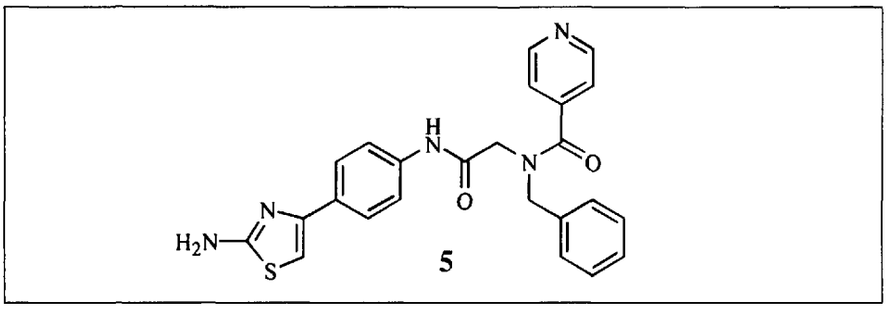
Helicase-Primase Inhibitors as Novel Anti-HSV Agents

Bruno Simoneau, *Boehringer* Ingelheim (Canada) Ltd., Bio-Méga Research Division

Epidemiology of Herpes Simplex Virus Infections. HSV Type-1 is responsible for labial herpes, whereof one out of every two individuals worldwide is affected, and herpes-induced keratitis which is the leading cause of infectious blindness with 300'000 new cases per year. HSV Type-2 causes genital herpes, a disease with 500'000 new cases per year. Both types are responsible for neonatal encephalitis with a mortality of 50%. Most significant is their capability for latent infection.

Current Therapies for HSV Infections. The nucleoside-based *Aciclovir* (*Valaciclovir*, ACV) and *Penciclovir* (*Famciclovir*) are currently used as therapeutics. Major indications of ACV are primary and recurrent genital herpes, herpes labialis, keratitis, and encephalitis. Its most significant deficits are the dosing frequency, rate of recurrence after primary infection, the slow onset of action and resistance in immunocompromised patients. Therefore, the antiviral research focuses on improvement of oral bioavailability (dosing) and different mechanisms of action (resistance).

HSV-1 Proteins Essential for DNA Replication. Seven proteins essential for DNA replication in HSV-1 have so far been identified and might be valuable targets for new antiviral drugs. Among those,



5

UL5, UL52, and UL8 are helicase, primase, and the helicase-primase accessory protein, respectively. They have been purified from infected cells as heterotrimeric complex and exhibit DNA-dependent ATPase, 5'-3' helicase and RNA-primase activities, whereas the recombinant (baculo) UL5/UL52 subassembly shows all three activities.

Assays to Evaluate Inhibitors. The DNA helicase is analyzed by an unwinding assay with radiolabeled M13 ssDNA which is after liberation hybridized to a biotin-labelled oligomer, which is collected with avidin beads and counted by its radiolabel. The DNA-dependent ATPase activity is detected in a set-up where, in presence of DNA, ATP is hydrolyzed and the developing ADP + P_i is detected with a colorimetric assay using malachite green. DNA-primase activity is measured by a biotin-labelled 50-mer which is primed with ^{3}H-labeled GTPs. The incorporated activity is counted after collection of the primed 50-mers on avidin beads.

Viral Replication Assays. The applied assays are a glycoprotein-based ELISA assay in 96-well format and a plaque-reduction assay in 12-well format.

Inhibitor Screening. Starting point for a selective HSV helicase-primase inhibitor was high-throughput screening which revealed 4-thiazolyl-2-thiazolamine, and the substructure search led to a benzylglycine amide derivative thereof with an EC_{50} (ELISA) of 2.6 mM. Further derivatives were screened and led to BILS 45, an inhibitor selected for evaluation in animal models of HSV disease.

EC_{50} values of *in vitro* antiviral activity of BILS 45 are around 0.15 mM, also against several HSV-1, -2 and ACV^r strains. The mode of action is confirmed by several experiments indicating that helicase-primase is the target of antiviral action.

Conclusion. Substituted 4-phenyl-2-thiazolamine derivatives have been identified as potent selective inhibitors of HSV-1 helicase-primase. The inhibitors act competitively with respect to ATP and stabilize the interaction of the enzyme with DNA. Furthermore, *in vitro* helicase-primase inhibitors arrest growth of wild-type and ACV^r HSV-1 and HSV-2 while being orally active. Therefore, inhibition of helicase-primase may be an effective strategy to generate novel clinically useful antiherpetic agents.

Multidrug-Resistance Systems in *Lactococcus lactis*

Wilhelm N. Konings, Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, Kerklaan 30, NL-9751 NN, Haren, The Netherlands

The lactic acid bacterium *Lactococcus lactis* possesses two multidrug-resistance transporters with specificity for lipophilic compounds. One system, LmrP, belongs to the Major Facilitator Superfamily and is driven by the proton-motive force. The other system, LmrA, belongs to the ATP-binding cassette (ABC) superfamily and is driven by ATP hydrolysis. The genes encoding these transport proteins were identified and expressed in *Escherichia coli*. Methods have been developed to routinely overexpress histidine-tagged LmrP and LmrA in *L. lactis*, for LmrA up to 35% of the total membrane protein. For this expression, a novel protein-expression system for cytotoxic proteins was used which was based on the nisin-inducible nisA promoter. LmrP and LmrA were efficiently solubilized with dodecyl maltoside (DDM), purified by Ni-affinity, and reconstituted in DDM-destabilized preformed liposomes prepared from *E. coli* phosholipids and egg phosphatidylcholine. The functional properties of both proteins were studied in whole cells, membrane vesicles, and proteoliposomes.

The substrate specificity of LmrP and LmrA was found to be very similar to that of the human multidrug-resistance P-glycoprotein (Pgp). A detailed analysis of the mechanism(s) involved in drug excretion revealed that both LmrP and LmrA pick up the cytotoxic compounds from the inner leaflet of the plasma membrane and excrete the compounds directly into the external medium. This ingenious 'vacuum-cleaner' mechanism ensures that the cytotoxic compounds cannot reach the cytosol to exert their toxic effects. By using *Hoechst 33342*, which is highly lipophilic and fluorescent only in the lipid bilayer, as a substrate for LmrP and LmrA, the kinetics of *Hoechst* transport and the inhibiting properties of a wide variety of substrates could be determined. These studies revealed a K_m for *Hoechst* of 1 molecule per 2000 lipid molecules.

LmrA contains an *N*-terminal membrane domain with six membrane-spanning segments followed by the ABC domain. LmrA is homologous to certain eukaryotic ABC transporters and is a half-molecule version of the human multidrug-resistance protein *P*-glycoprotein. To study the domain organization of LmrA, fusion proteins were constructed in which LmrA was linked to itself using the 'linker' peptide of Pgp. ATPase activity and drug-transport measurements revealed that the dimer is active. In contrast, dimeric LmrA proteins containing a Lys-to-Met substitution at position 388 in the *Walker* A region of the first and/or the second ABC domain have lost the ability to transport drugs or hydrolyze ATP. These results suggest that LmrA functions as a homodimer in which ATP hydrolysis by both ABC domains is required.

An analysis of vinblastine binding to LmrA suggests the presence of two allosterically linked drug-binding sites. Posi-