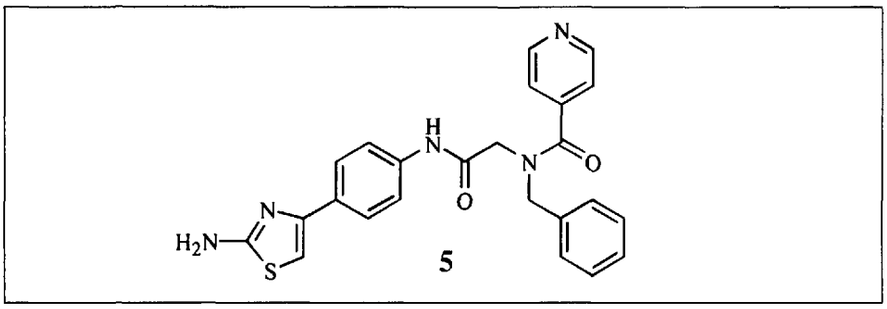

5

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-

tive cooperativity of vinblastine binding was observed. The presence of two vinblastine-binding sites is further supported by *i*) heterologous displacement experiments in which vinblastine bound by LmrA was displaced by the inhibitor CP 100-356 in a biphasic fashion, and *ii*) stoichiometric determinations which reveal the binding of two vinblastine molecules per LmrA transporter. Trapping of LmrA in the ADP/vanadate-bound transition-state conformation resulted in reduced efficiency of photo-affinity labelling with (3H)APDA, indicating that drug binding and ATP hydrolysis are coupled. Vinblastine-displacement experiments and vinblastine/transporter stoichiometry determinations suggest that the ADP/vanadate-trapped transporter contains a single low-affinity drug-binding site.

These observations have been incorporated in a sequential two-site mechanistic model of the LmrA transporter. It is obvious from the observations described above that LmrA is not only a structural but also a functional homologue of P-glycoprotein. To test that this is indeed the case, LmrA was functionally expressed in insect cells, oocytes, and human fibroblast cells. The analysis of the pharmacological properties of LmrA in these heterologous expression systems showed that LmrA and P-glycoprotein behave very similar.

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- B.Z. Scholten, Microbiology, University of Groningen, P.O. Box 14, NL–9750 AA HAREN, tel +31 503 63 21 50, fax +31 503 63 21 54.

Modulation of Multidrug Resistance in Cancer Cells by Inhibitors of P-Glycoprotein

Ahmad R. Safa, Department of Experimental Oncology, Hollings Cancer Center, Medical University of South Carolina, P.O. Box 250955, Charleston, SC 29425, USA

Most metastatic cancers are either intrinsically resistant to chemotherapy or acquire drug resistance subsequent to chemotherapy. Therefore, a major problem in cancer chemotherapy is the development of resistance to chemotherapeutic agents in tumor cells.

In vitro selection of cancer cells for resistance to lipophilic cytotoxic agents usually results in the development of cross-resistance to many other drugs which share little structural similarity. These drugs include *Vinca* alkaloids, anthracyclins, epipodophyllotoxins, taxol, actinomycin D, colchicine, puromycin, gramicidin D_1, and others. The hallmark of this type of multidrug resistance is overexpression of the MDR1-gene product, a 170 kDa phosphoglycoprotein termed P-glycoprotein (P-gp), which is involved in the efflux of these agents. We and others have shown that many structurally distinct agents reverse multidrug resistance (MDR) by binding to P-gp and inhibiting the efflux of the MDR-related cytotoxic agents. However, the precise mechanism of how these agents interact with P-gp and reverse MDR is not well understood.

To investigate how the MDR-related cytotoxic agents bind to P-gp and inhibits effluxes, and whether there is a direct relationship between MDR reversal and modulator interaction at the level of P-gp inhibition, we have used five approaches including *1*) reversing MDR in drug-resistant cell lines by using potent MDR modulators, *2*) performing drug-accumulation studies in drug-sensitive and MDR variants, *3*) synthesizing photoaffinity analogs of the MDR-related cytotoxic agents and modulators, and identifying and characterizing their binding sites by photoaffinity labeling, proteolytic digestion, and using P-gp epitope-specific antibodies, *4*) inhibiting vinblastine, doxorubicin, verapamil, and azidopine by photolabeling of P-gp, and *5*) performing kinetic analysis and inhibition of binding of radioactive vinblastine and vincristine to plasma-membrane vesicles under equilibrium conditions. Using these approaches, we have found that potent modulators of P-gp interact either competitively or allosterically with the cytotoxic-drug-binding sites of P-gp and inhibit its function, thereby allowing the cytotoxic agent to accumulate in the MDR cells, inhibit growth, and cause cell death. Among diverse classes of modulators, we have found that cyclosporin A and its non-immunosuppressive analog PSC 833, FK-506, two diaminoquinazolines CP100356 and CP114476, the quinoline derivative CP117227, tamoxifen, several reserpine and yohimbine derivatives, megestrol acetate, and phenothiazines and related compounds, particularly *trans*-flupentixol and iodoazidophenethylspiperone (I-NAPS) are effective inhibitors of P-gp.

Our results using these modulators demonstrate a direct relationship between the reversal of MDR and their interaction with the drug-binding sites of P-gp. Moreover, our data revealed that vinblastine, verapamil, and azidopine bind to at least one common binding domain located within or immediately *C*-terminal to transmembrane domain 6 (TMD 6) of P-gp. However, kinetic analysis revealed that vinblastine and cyclosporin A competitively interact for the same binding site while they non-competitively interact with the azidopine-binding site. Therefore, these results suggest that vinblastine and azidopine bind to separate binding sites on a common domain. Interestingly, deletion of a phenylalanine at position 335 of P-gp (located in TMD 6) has been shown to cause resistance to cyclosporin A and PSC 833. Our data provide direct evidence that TMD 6 is an important domain of P-gp for recognition of some MDR-related drugs and potent P-gp inhibitors.