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## Study of the Mechanism of Antiviral Action of Iminosugar Derivatives against Bovine Viral Diarrhea Virus

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**The glucose-derived iminosugar derivatives *N*-butyl- and *N*-nonyl-deoxyojirimycin (DNJ) have an antiviral effect against a broad spectrum of viruses including *Bovine viral diarrhea virus* (BVDV). For BVDV, this effect has been attributed to the reduction of viral secretion due to an impairment of viral morphogenesis caused by the ability of DNJ-based iminosugar derivatives to inhibit ER  $\alpha$ -glucosidases (N. Zitzmann, A. S. Mehta, S. Carrouée, T. D. Butters, F. M. Platt, J. McCauley, B. S. Blumberg, R. A. Dwek, and T. M. Block, *Proc. Natl. Acad. Sci. USA* 96:11878–11882, 1999). Here we present the antiviral features of newly designed DNJ derivatives and report for the first time the antiviral activity of long-alkyl-chain derivatives of deoxygalactonojirimycin (DGJ), a class of iminosugars derived from galactose which does not inhibit endoplasmic reticulum (ER)  $\alpha$ -glucosidases. We demonstrate the lack of correlation between the ability of long-alkyl-chain DNJ derivatives to inhibit ER  $\alpha$ -glucosidases and their antiviral effect, ruling out ER  $\alpha$ -glucosidase inhibition as the sole mechanism responsible. Using short- and long-alkyl-chain DNJ and DGJ derivatives, we investigated the mechanisms of action of these drugs. First, we excluded their potential action at the level of the replication, protein synthesis, and protein processing. Second, we demonstrated that DNJ derivatives cause both a reduction in viral secretion and a reduction in the infectivity of newly released viral particles. Long-alkyl-chain DGJ derivatives exert their antiviral effect solely via the production of viral particles with reduced infectivity. We demonstrate that long-alkyl-chain DNJ and DGJ derivatives induce an increase in the quantity of E2-E2 dimers accumulated within the ER. The subsequent enrichment of these homodimers in secreted virus particles correlates with their reduced infectivity.**

Iminosugar derivatives containing the glucose analogue deoxyojirimycin (DNJ) exert antiviral effects against viruses of different families, including *Human immunodeficiency virus* (HIV) (9–12, 16), *Hepatitis B virus* (HBV) (3, 18, 19), *Woodchuck hepatitis virus* (4), *Bovine viral diarrhea virus* (BVDV) (29), and *Dengue virus* (8). The antiviral effects were either proven (10, 11) or assumed to be associated with the inhibitory action of DNJ-containing iminosugar derivatives on enzymes in the endoplasmic reticulum (ER),  $\alpha$ -glucosidases I and II. DNJ, a ring-nitrogen-containing and unmetabolizable glucose analogue, competitively binds to these enzymes and prevents them from performing the stepwise removal of three glucose residues attached to the N-linked glycans carried by newly synthesized polypeptides. This in turn prevents these polypeptides from interacting with the ER chaperones calnexin and calreticulin, which bind to monoglucosylated glycoproteins (14). Interaction with these ER chaperones is crucial for the correct folding of some but not all glycoproteins (13, 17, 22). Potentially all viruses which encode glycoproteins that depend on calnexin interaction for proper folding could be targeted using ER  $\alpha$ -glucosidase inhibitors, with those budding from the ER (e.g., HBV and members of the *Flaviviridae*) probably being most sensitive (20).

In the absence of a reliable cell culture system able to support hepatitis C virus (HCV) replication and based on the degree of homology in terms of genomic organization, replication strategy (including chronicity), and protein function (25), BVDV has been proposed as a surrogate model to study the action of antiviral molecules (2, 29). We have previously shown that the BVDV envelope glycoproteins E1, E2, and the E2 precursor E2-p7 interact with calnexin and that this interaction can be inhibited using the iminosugar derivative *N*-butyl-DNJ (NB-DNJ) (5). The antiviral effect observed using the ER  $\alpha$ -glucosidase inhibitors NB-DNJ and *N*-nonyl-DNJ (NN-DNJ) was attributed to the reduction of viral secretion due to an impairment of viral morphogenesis caused by  $\alpha$ -glucosidase inhibition (29). However, a precise study of the mechanism of action at the molecular level has yet to be performed.

In this report we demonstrate that in addition to the antiviral effect caused by ER  $\alpha$ -glucosidase inhibition, iminosugar derivatives carrying longer alkyl side chains have a second mechanism of action. We further report the antiviral activity of long-alkyl-chain deoxygalactonojirimycins (DGJ), a class of iminosugars derived from galactose which lack ER  $\alpha$ -glucosidase inhibitory activity. Using both DNJ- and DGJ-containing iminosugar derivatives, we investigated their mechanism of action and eliminated the possibilities that they act at the level of replication, protein synthesis, or protein processing. On the basis of the effect of the length of the alkyl chain attached to the carbohydrate analogue head group on viral morphogenesis, secretion, and infectivity, we propose a second mechanism

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of action involved in the antiviral activity of long-alkyl-chain iminosugar derivatives. Finally, we show that the abnormal accumulation of E2-E2 dimers in the ER, induced by treatment with long-alkyl-chain iminosugars, and its subsequent enrichment in the virus particles correlate with reduced infectivity of these particles.

## MATERIALS AND METHODS

**Cells, viruses, and inhibitors.** Madin-Darby bovine kidney (MDBK) cells were maintained at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere in RPMI 1640 medium (GIBCO/BRL) supplemented with 10% screened BVDV-free fetal calf serum (PAA Laboratories, Teddington, United Kingdom). The noncytopathic (ncp) Pe515 strain (23) and the cytopathic (cp) NADL (National Animal Disease Laboratory) strain were used in this study. The titers of the stock solutions of these viruses were determined to be  $4 \times 10^6$  and  $2 \times 10^7$  PFU/ml, respectively. NB-DNJ was a gift from Searle/Monsanto. NN-DGJ was purchased from Toronto Research Chemicals. 6-Deoxy-DGJ was kindly provided by G. W. Fleet (University of Oxford). NN-DNJ, N-nonyl-6-deoxy-DGJ, N7-oxadecyl-DNJ and N7-oxanonyl-6-deoxy-DGJ were either synthesized in-house or provided by Synergy Pharmaceuticals Inc.

**Synthesis and purification of N-alkylated DNJ and DGJ compounds.** N-alkylated-DNJ and -DGJ compounds were synthesized using sodium cyanoborohydride (NaBH<sub>3</sub>CN) and the appropriate aldehyde (21, 29). The C<sub>16</sub> and C<sub>18</sub> compounds were prepared using *cis*-11-hexadecanal and *cis*-13-octadecanal, respectively, and purified as described previously (21).

**MTS cell proliferation assay.** MDBK cells were grown and incubated on 96-well plates at 37°C in the presence of different concentrations of inhibitors (in triplicate) for 3 days. The number of proliferating cells was determined using the CellTiter 96 AQueous nonradioactive cell proliferation assay (Promega, Southampton, United Kingdom) as specified by the manufacturer. Cells were incubated with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-phenazine methosulfate (PMS) solution for 3 h at 37°C before the absorbance at 490 nm was read using an enzyme-linked immunosorbent assay plate reader. The 50% cytotoxic concentration (CC<sub>50</sub>) was determined as the drug concentration at which half of the cells were not proliferating compared to untreated control cells.

**BVDV plaque reduction assay.** MDBK cells were grown in six-well plates to 90% confluency and infected with cp BVDV (at a multiplicity of infection [MOI] of 0.01, 0.1, or 1) for 1 h at 37°C. After removal of the inoculum, the cells were washed with phosphate-buffered saline (PBS) and incubated in RPMI 1640 medium containing 10% fetal calf serum and inhibitors at different concentrations. After 2 or 3 days, the medium containing secreted virus was removed from the wells and centrifuged at low speed to remove cellular debris. Different dilutions were used to infect a fresh monolayer of MDBK cells in six-well plates. After 2 days, the resulting plaques were counted. The 50 and 90% inhibitory concentrations (IC<sub>50</sub>) or (IC<sub>90</sub>) were determined as the concentrations at which the number of plaques was halved or reduced by 90%, respectively, compared to untreated infected control cells.

**Analysis of viral genome synthesis.** Subconfluent (90% confluent) MDBK cell monolayers grown in 25-cm<sup>2</sup> flasks were infected with BVDV at a MOI of >5. After 1 h, the inoculum was removed and the cells were washed twice in PBS before 5 ml of fresh medium containing inhibitors at the concentrations indicated was added. The inhibitors were present throughout the experiment. At 6 h postinfection (h p.i.), actinomycin D (5 µg/ml) was added to the medium to block host RNA synthesis and kept present for the rest of the experiment. At 8 h p.i., RNA was labeled by the addition of 100 µCi of [5,6-<sup>3</sup>H]uridine per ml to the medium. At 6 h after the onset of labeling, total RNA was extracted using the RNeasy purification kit (Qiagen, Crawley, United Kingdom) as specified by the manufacturer. RNA was fractionated by agarose-formaldehyde gel electrophoresis and transferred by capillary action onto Hybond N<sup>+</sup> membrane (Amersham, Little Chalfont, United Kingdom) as described by Ausubel et al. (1). The membrane was treated with a fluorographic reagent (Amersham) for 30 min, dried, and exposed to Hyperfilm-MP (Amersham) at -70°C for 14 days. The intensity of the bands on the resulting autoradiogram was measured by scan densitometry.

**Viral RNA purification and RT-PCR analysis.** MDBK cells (8 × 10<sup>5</sup>/35-mm-diameter dish) were infected at an MOI of 1 with either cp or ncp BVDV. After 1 h, the inoculum was removed and the cells were washed twice in PBS before 2 ml of fresh medium containing inhibitors at the concentrations indicated was added. At 24 or 48 h p.i., the supernatant was harvested, centrifuged at 5,000 × g for 5 min, and stored at -70°C before being used for viral RNA purification. RNA from released viral particles was purified using the QIAamp viral RNA purification kit (Qiagen) with 140 µl of supernatant as the starting material.

Reverse transcription-PCR (RT-PCR) was performed using the Titan One Tube RT-PCR system (Roche) and two primers, P1 (5'-AACAAACATGGTTGGTGCAACTGGT-3') and P2 (5'-CTTACACAGACATATTTGCCTAGGTTCCA-3'), which amplify a 826-bp region overlapping E<sup>7ns</sup> and E1 (27). A total of 35 cycles (10 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 68°C and 25 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min plus 5 s/cycle at 68°C) of PCR were performed. The PCR products were separated using 2% agarose gels stained with ethidium bromide.

**Protein extraction, SDS-PAGE, and Western blot analysis.** MDBK cells (8 × 10<sup>5</sup>/35-mm-diameter dish) were infected at an MOI of 1 with either cp or ncp BVDV. After 1 h, the inoculum was removed and the cells were washed twice in PBS before 2 ml of fresh medium containing inhibitors at the concentrations specified was added. At 24 or 48 h p.i., the cells were harvested by trypsin-EDTA treatment, washed once with PBS, centrifuged at 5,000 × g for 5 min, and stored as a dry pellet at -70°C before being used for protein extraction. Cell lysis was performed at 4°C for 1 h in CHAPS-HSE buffer (2% CHAPS, 50 mM HEPES [pH 7.5], 200 mM NaCl, 2 mM EDTA) in the presence of a cocktail of protease inhibitors (Sigma, St. Louis, Mo.) and 20 mM iodoacetamide (Sigma). Iodoacetamide alkylates free sulfhydryl groups and avoids nonspecific disulfide bond formation. The protein concentration of the cell lysates was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). Protein samples were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing or reducing conditions and transferred onto nitrocellulose membranes. Three monoclonal antibodies (MAbs) directed against the BVDV E2 protein (MAbs WB214, WB166, and WB158 [Veterinary Laboratory Agency, Weybridge, United Kingdom]) were used as primary antibodies in this study. MAbs WB214 and WB166 recognize a linear epitope and are used under both nonreducing and reducing conditions. Mab WB158 is a conformation-dependent antibody, which recognizes E2 only under nonreducing conditions. Peroxidase-conjugated sheep anti-mouse immunoglobulin G (Amersham) was used as secondary antibody (diluted 1:2,000 in PBS). Proteins were detected using the ECL system as specified by the manufacturer (Amersham).

**Pulse-chase labeling and immunoprecipitation.** Subconfluent MDBK cell monolayers grown in 25-cm<sup>2</sup> flasks were infected with BVDV at an MOI of 1. After a 1-h incubation at 37°C, the viral inoculum was replaced with medium containing 10% FCS. At 18 h p.i., monolayers were washed once with PBS and incubated in methionine- and cysteine-free RPMI 1640 medium (ICN Flow). After 1 h, the cells were pulse-labeled with 100 µCi of [<sup>35</sup>S]methionine-[<sup>35</sup>S]cysteine (Tran <sup>35</sup>S-label, 1,100 Ci/mmol; ICN Flow) per ml at 37°C for the times indicated. Following labeling, the isotope-supplemented medium was removed and the cells were washed once with PBS and chased for various times in RPMI 1640 medium containing 10 mM unlabeled methionine. At the time points indicated, the chase media were discarded and the cells were harvested. When the effect of the iminosugar derivative on protein synthesis and protein processing was investigated, the drug was added to the cells at the concentrations indicated 2 h before the pulse and was present throughout the chase period. The cells were then lysed for 1 h on ice in a buffer containing 0.5% Triton X-100, 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 2 mM EDTA (Triton-TSE buffer), and a mixture of protease inhibitors. Labeled cell lysates were clarified by centrifugation at 12,000 × g for 15 min and precleared with 20 µl of protein G-Sepharose for 1 h at 4°C. The lysates were briefly centrifuged, and the supernatants were incubated with anti-BVDV E2 (MAb WB214; 1:50 dilution) overnight at 4°C. Protein G-Sepharose (30 µl) was then added to the supernatants, and the incubation was continued for 1 h at 4°C. The slurry was washed six times with 0.2% Triton X-100 in TSE buffer. The immunoprecipitated complexes were eluted by boiling the samples for 10 min in Laemmli buffer and separated by SDS-PAGE. After electrophoresis, the gels were treated with Amplifier (Amersham), dried, and exposed at -70°C to Hyperfilm-MP (Amersham).

## RESULTS

**Antiviral features of newly developed iminosugar derivatives: importance of the head group and alkyl chain length.** NB-DNJ and the longer-alkyl-side-chain-containing NN-DNJ have been reported previously to show an antiviral effect against BVDV in vitro (29). These experiments were performed using a low MOI (0.01). Since the MOI which is relevant with respect to an in vivo situation is unknown, we decided to investigate whether these molecules had antiviral properties at higher MOIs. We evaluated the antiviral effect of these two drugs and other iminosugar derivatives (Fig. 1) in BVDV

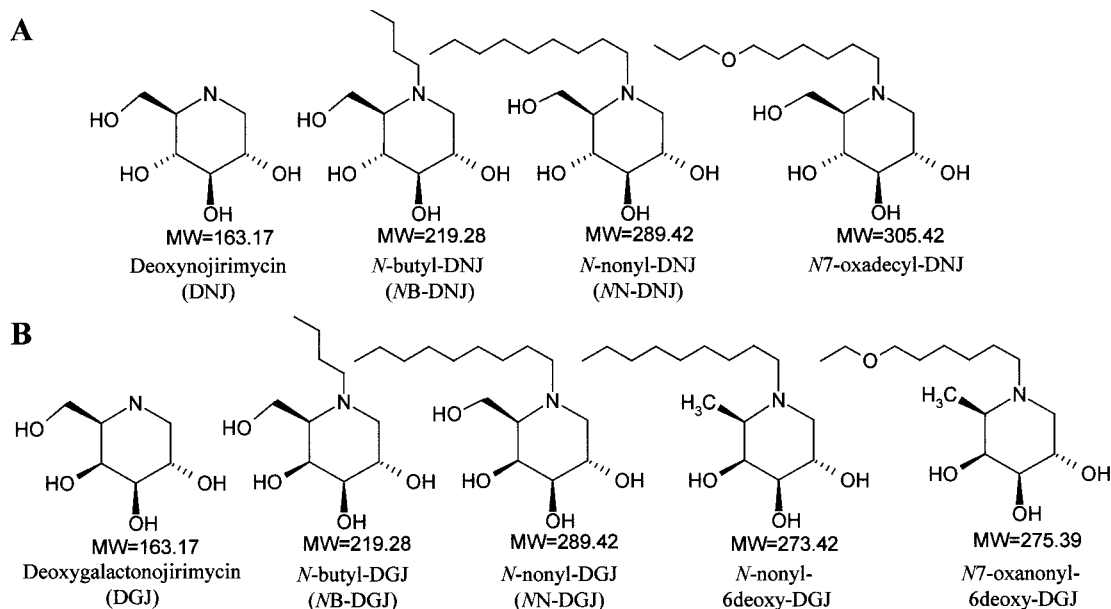


FIG. 1. Chemical structures of the iminosugar derivatives used in this study. (A) Iminosugar derivatives based on the glucose analogue DNJ. (B) Iminosugar derivatives based on the galactose analogue DGJ.

plaque reduction assays using MDBK cells infected at different MOI (0.01, 0.1, and 1) (Table 1). The toxicity of the drugs determined in MTS assays was used to calculate the in vitro selectivity index ( $CC_{50}/IC_{50}$ ) (Table 2). Introduction of an oxygen atom at position 7 of the alkyl side chain led to a major reduction in toxicity and significant improvement of the selectivity index.

NN-DNJ is at least 10 times more potent than NB-DNJ in BVDV plaque reduction assays. Since both inhibit ER  $\alpha$ -glucosidase and since NB-DGJ is not antiviral at all (29), this difference in potency had been attributed to the difference in alkyl chain length. To assess the influence of the alkyl chain length on in vitro drug potency, a series of compounds with alkyl chains of different lengths ranging from  $C_4$  to  $C_{18}$ , attached to the DNJ head group, was synthesized and screened for antiviral activity in the BVDV plaque reduction assay (Fig. 2). The compounds in the middle of the range ( $C_8$  to  $C_{10}$ -DNJ) were most efficient, with NN-DNJ being the most potent inhibitor ( $IC_{50[MOI\ 0.01]} = 2.5\ \mu M$ ). This difference in antiviral potency could be due to differences in cellular uptake and intracellular distribution. To assess whether the increased po-

tency was due to a detergent-like effect of the longer alkyl chains, we tested the detergents *n*-octyl- and *n*-nonylglucoside, as well as nonylamine and NN-DGJ. While *N*-octyl- and *N*-nonylglucoside and nonylamine were not antiviral at all (data not shown), ruling out a detergent-like effect, the longer-alkyl-side-chain-containing NN-DGJ showed antiviral activity. The  $IC_{50}$ s obtained with NN-DGJ were comparable to those obtained with NN-DNJ (Table 1). NN-DGJ does not inhibit ER  $\alpha$ -glucosidases, but it is an inhibitor of the ceramide-specific glucosyltransferase, an enzyme involved in glycolipid biosynthesis (reviewed in reference 6). However, since the shorter-alkyl-chain derivative NB-DGJ has been shown to be totally ineffective in antiviral assays at concentrations which were sufficient to completely inhibit the ceramide-specific glucosyltransferase (29), the antiviral effect observed with NN-DGJ cannot be explained by the inhibition of the glycolipid biosynthesis pathway. This was confirmed by another compound, NN-6deoxy-DGJ, which, due to the methyl group modification at position 5 of the carbohydrate ring (Fig. 1), is a poor inhibitor of the ceramide-specific glucosyltransferase (T. Butters,

TABLE 1. Antiviral features of different iminosugar derivatives

Compound	$IC_{50}$ ( $\mu M$ ) <sup>a</sup> at MOI of:			$IC_{90}$ ( $\mu M$ ) <sup>a</sup> at MOI of:		
	0.01	0.1	1	0.01	0.1	1
DNJ	22.5 $\pm$ 2.5	ND	ND	125 $\pm$ 25	ND	ND
DGJ	NO	NO	NO	NO	NO	NO
NB-DNJ	27.5 $\pm$ 2.5	125 $\pm$ 25	150 $\pm$ 50	175 $\pm$ 25	600 $\pm$ 100	1,000 $\pm$ 100
NB-DGJ	NO	NO	NO	NO	NO	NO
NN-DNJ	2.5 $\pm$ 0.5	10 $\pm$ 2.5	12.5 $\pm$ 2.5	20 $\pm$ 5	35 $\pm$ 5	85 $\pm$ 5
NN-DGJ	2.5 $\pm$ 0.5	7.5 $\pm$ 2.5	25 $\pm$ 5	7.5 $\pm$ 2.5	NR	NR
NN-6deoxy-DGJ	2 $\pm$ 0.5	6.5 $\pm$ 1.5	25 $\pm$ 5	<7.5	NR	NR
N7-oxadecyl-DNJ	17.5 $\pm$ 2.5	35 $\pm$ 5	80 $\pm$ 20	62.5 $\pm$ 12.5	200 $\pm$ 20	1,000 $\pm$ 200
N7-oxanonyl-6deoxy-DGJ	2.5 $\pm$ 0.5	17.5 $\pm$ 2.5	125 $\pm$ 25	7.5 $\pm$ 2.5	NR	NR

<sup>a</sup> Values indicated are the means of numbers obtained in at least three sets of experiments  $\pm$  standard deviation. ND, not determined; NO, not antiviral; NR, not reached.



TABLE 2. Toxicity and selectivity index of different iminosugar derivatives

Compound	CC <sub>10</sub> (μM) <sup>a</sup>	CC <sub>30</sub> (μM)	CC <sub>50</sub> (μM)	SI50 (CC <sub>50</sub> /IC <sub>50</sub> ) at MOI of:		
				0.01	0.1	1
DNJ	>1,000	>2,000	>5,000	>200	ND	ND
DGJ	ND <sup>b</sup>	ND	>10,000	NA <sup>c</sup>	NA	NA
NB-DNJ	ND	>10,000	>10,000	>333.33	>66.66	>50
NB-DGJ	ND	ND	ND	NA	NA	NA
NN-DNJ	50 ± 10	187.5 ± 12.5	237.5 ± 12.5	>75	>18	>15
NN-DGJ	112.5 ± 12.5	200 ± 10	237.5 ± 12.5	>75	>22.5	>7.5
NN-6deoxy-DGJ	75 ± 5	100 ± 20	187.5 ± 12.5	>70	>21.875	>5.833
N7-oxadecyl-DNJ	>1,000	>2,000	>5,000	>250	>125	>50
N7-oxanonyl-6deoxy-DGJ	>3,000	>3,500	>4,000	>1,333.33	>200	>26.66

<sup>a</sup> CCs were obtained as described in Materials and Methods. Values indicated are the means of numbers obtained in at least three sets of experiments ± standard deviation.

<sup>b</sup> ND, not determined.

<sup>c</sup> NA, not available.

unpublished data), but still gave rise to IC<sub>50</sub>s comparable to those reached with NN-DNJ and NN-DGJ in the BVDV plaque reduction assay (Table 1). These results indicate a novel mechanism of action responsible for the antiviral effect of longer-alkyl-chain DGJ derivatives. This mechanism of action, which is associated with the length of the alkyl side chain, also contributes to the antiviral effect of DNJ derivatives, which so far has been attributed to the inhibition of the ER α-glucosidases alone.

To confirm the latter point, we analyzed the correlation of the antiviral effect of DNJ derivatives and the extent of ER α-glucosidase inhibition achieved. BVDV-infected cells were treated with NB-DNJ and NN-DNJ at IC<sub>50</sub> and IC<sub>90</sub>. The migration pattern of the intracellular viral envelope glycoprotein E2 and its precursor E2-p7 in treated and untreated cells was analyzed by SDS-PAGE and Western blotting (Fig. 3). The upward shift of E2 and E2-p7 in samples treated with DNJ-based inhibitors was due to hyperglucosylated structures accumulating as a result of ER α-glucosidase inhibition. The shift could be reversed by incubating inhibitor-treated samples with ER α-glucosidases I and II (data not shown). At the re-

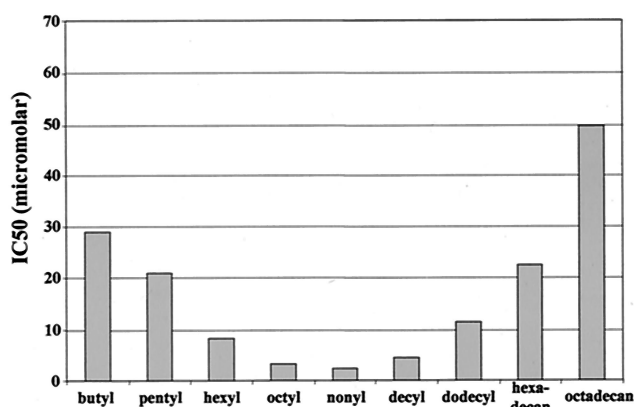


FIG. 2. Antiviral effect of DNJ derivatives in the “in vitro” BVDV system. DNJ derivatives containing alkyl side chains of different lengths ranging from C<sub>4</sub> to C<sub>18</sub> (x axis) were tested for their ability to inhibit BVDV plaque formation on MDBK cells as described in Materials and Methods. An MOI of 0.01 was used. Hexadecan-DNJ and octadecan-DNJ are unsaturated. The IC<sub>50</sub> of each molecule is shown on the y axis.

spective IC<sub>50</sub> and IC<sub>90</sub>, this shift was more pronounced for the shorter-alkyl-side-chain-containing NB-DNJ than for the longer-alkyl-side-chain-containing NN-DNJ, demonstrating an obvious lack of correlation between the extent of glucosidase inhibition and the antiviral activity observed for NN-DNJ. This observation led us to further investigate the second mechanism of action associated with the length of the alkyl side chain.

**Iminosugars do not inhibit viral RNA synthesis or the formation and processing of the viral polyprotein.** Generally it has been assumed that DNJ derivatives exert their antiviral activity via α-glucosidase inhibition only. We have shown that this is not the case for long-alkyl-chain DNJ derivatives and that an additional and as yet undefined mechanism is involved. It was therefore necessary to establish whether this second mechanism could target either BVDV RNA synthesis or the formation and processing of the BVDV polyprotein. The impact of the long-alkyl-chain-containing NN-DNJ and NN-DGJ, representing the two classes of iminosugar derivatives used in this study, on viral RNA synthesis was studied by monitoring the incorporation of [5,6-<sup>3</sup>H]uridine into newly synthesized BVDV genomic RNA in the presence or absence of the inhibitors. To this end, we used the method described previously by Purchio et al. (24) (see Materials and Methods). The amount of rRNA (28S and 18S) present in each lane was quantified and used to normalize the quantity of radio-labeled viral RNA. Neither NN-DNJ nor NN-DGJ treatment led to a significant

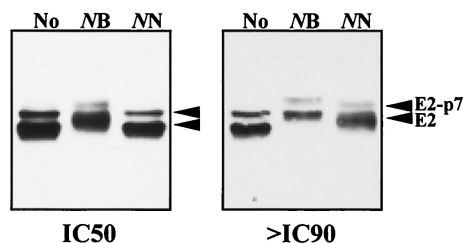


FIG. 3. ER α-glucosidase inhibition and antiviral effect of iminosugar derivatives. BVDV-infected MDBK cells (MOI of 1) were not treated (No) or were treated with NB-DNJ (NB) and NN-DNJ (NN) at their respective IC<sub>50</sub>s (left panel) and IC<sub>90</sub>s (right panel). Cells were lysed at 18 h p.i., and proteins were separated by SDS-PAGE (10% polyacrylamide) under reducing conditions. A Western blot analysis was performed using the anti-E2 MAb WB214 (diluted 1:1,000). The two polypeptides detected (E2 and E2-p7) are indicated by arrows.

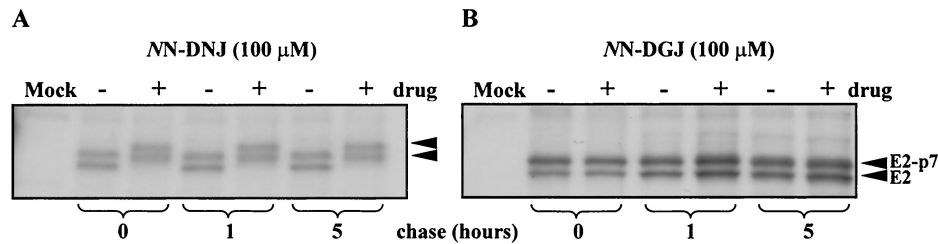


FIG. 4. Biosynthesis and processing of BVDV envelope glycoproteins in the presence of *NN-DNJ* or *NN-DGJ*. MDBK cells were infected with BVDV at an MOI of 1. At 18 h p.i., the cells were not treated (–) or were treated (+) with 100 μM of *NN-DNJ* (A) or *NN-DGJ* (B). At 2 h later, the cells were pulse-labeled with [<sup>35</sup>S]methionine-[<sup>35</sup>S]cysteine for 15 min, chased for the times indicated in the continuous presence of the drug, and immunoprecipitated with anti-E2 MAb WB214. The proteins were separated by SDS-PAGE (10% polyacrylamide) under reducing conditions and visualized by autoradiography. The two main polypeptides detected (E2 and E2-p7) are indicated by arrows.

reduction in the amount of radiolabeled genomic viral RNA, whereas control treatment with ribavirin, an inhibitor of viral replication, caused a significant reduction in the quantity of radiolabeled viral RNA detected (data not shown).

Having ruled out a direct influence of the drugs on viral RNA replication, we investigated their potential impact on viral protein synthesis and processing. BVDV translates its single large open reading frame into a polyprotein precursor, which is subsequently cleaved by a range of host cell and virally encoded proteinases to give rise to nonstructural and structural proteins (25). The structural proteins include the three envelope glycoproteins E<sup>trns</sup>, E1, and E2 (and its uncleaved precursor, E2-p7). For our investigations we chose E2 since we have studied its synthesis and chaperone-assisted folding in detail (5) and since MAbs recognizing all forms of E2 are available. MAb WB214 binds to E2 (and E2-p7) in all states of folding, under reducing and nonreducing conditions. We measured the level of E2 synthesis in the presence and absence of 100 μM *NN-DNJ* and *NN-DGJ*, respectively, in a pulse-chase experiment followed by immunoprecipitation with MAb WB214. The two bands corresponding to E2 and E2-p7 glycoproteins were detected throughout the chase period in both untreated and treated samples (Fig. 4). Treatment with *NN-DNJ* resulted in an upward shift of the glycoprotein bands (Fig. 4A), since the inhibition of N-linked glycan trimming leads to hyperglucosylation of the proteins. As expected, this shift was not observed in *NN-DGJ*-treated samples (Fig. 4B), since DGJ derivatives are not recognized by and hence are not inhibitors of ER α-glucosidases. Neither *NN-DNJ* nor *NN-DGJ* affected viral protein synthesis, and no significant difference in band intensity was observed between untreated and treated samples for up to 5 h of chase. Furthermore, processing of the polyprotein precursor into the final products (only E2 and E2-p7 are shown here) is not impaired by the presence of either drug. Taken together, these results indicate that iminosugar derivatives affect a part of the virus life cycle occurring after viral RNA synthesis and after polyprotein formation and processing.

**DNJ but not DGJ derivatives impair the secretion of virus particles.** Previously we assumed that all of the antiviral effect observed with *NB-DNJ* and *NN-DNJ* was due to the inhibition of secretion of virions into the culture medium (29). The experiments leading to this conclusion had been performed at an MOI of 0.01 in a 3-day plaque reduction assay, i.e., under conditions which were not optimal to study the secretion of viral RNA. We therefore analyzed the effects of both DNJ and

DGJ derivatives on the secretion of virions by using MDBK cells infected at a higher MOI. Cells infected with the cytopathic NADL strain of BVDV at an MOI of 1 were treated with both DNJ and DGJ derivatives. At 24 h p.i., the level of enveloped viral RNA released was measured by RT-PCR. The viral RNA was purified either directly from culture medium supernatants or from the virus-enriched fraction pelleted through a 20% sucrose cushion. To achieve accurate quantification by the end-point RT-PCR method, fivefold dilutions of the RNA samples were subjected to the same number of PCR cycles (15, 27). At the same time, an RT-PCR standard curve was obtained using supernatants obtained by twofold serial dilutions of a virus stock with a known titer of  $2 \times 10^7$  PFU/ml. The RT-PCR products were separated by agarose gel electrophoresis (2% agarose) and visualized by ethidium bromide staining (Fig. 5A). Densitometry analysis of the gel revealed a 5.5- and 4.5-fold reduction of the signal (compared to the untreated control) after *NB-DNJ* (2.5 mM) and *NN-DNJ* (100 μM) treatment, respectively. No reduction was observed after treatment with *NN-DGJ*. Identical results were obtained using RNA purified directly from the supernatant or purified from material pelleted through a 20% sucrose cushion, indicating that unenveloped RNA, which could have led to a misinterpretation of the result, was not present. These results indicate that DNJ but not DGJ derivatives impair the secretion of virus particles.

**Treatment with long-alkyl-chain DNJ derivatives and DGJ derivatives leads to the creation of viral particles with reduced infectivity, which contributes to the antiviral effect observed.** The standard curve in the experiment described above was used to determine the theoretical titer (by comparing the cDNA quantities of treated and untreated samples with those of serial dilutions of a virus stock of known titer) for each of the samples analyzed. This was then compared to the actual titer established in plaque reduction assays. The experimentally determined titers were  $7 \times 10^5$  PFU/ml for nontreated,  $6 \times 10^4$  PFU/ml for *NB-DNJ* treated,  $8 \times 10^4$  PFU/ml for *NN-DNJ*-treated, and  $1 \times 10^5$  PFU/ml for *NN-DGJ*-treated sample supernatants (table in Fig. 5A). This represented a 11.65-, 8.75-, and 7.1-fold drop in the titer for *NB-DNJ*-, *NN-DNJ*-, and *NN-DGJ*-treated samples, respectively. Compared to the theoretical titer (table in Fig. 5A), these numbers indicated a discrepancy between the quantity of viral RNA detected and the actual titer of infective virus. The results indicate that DNJ derivatives inhibit the secretion of viral particles

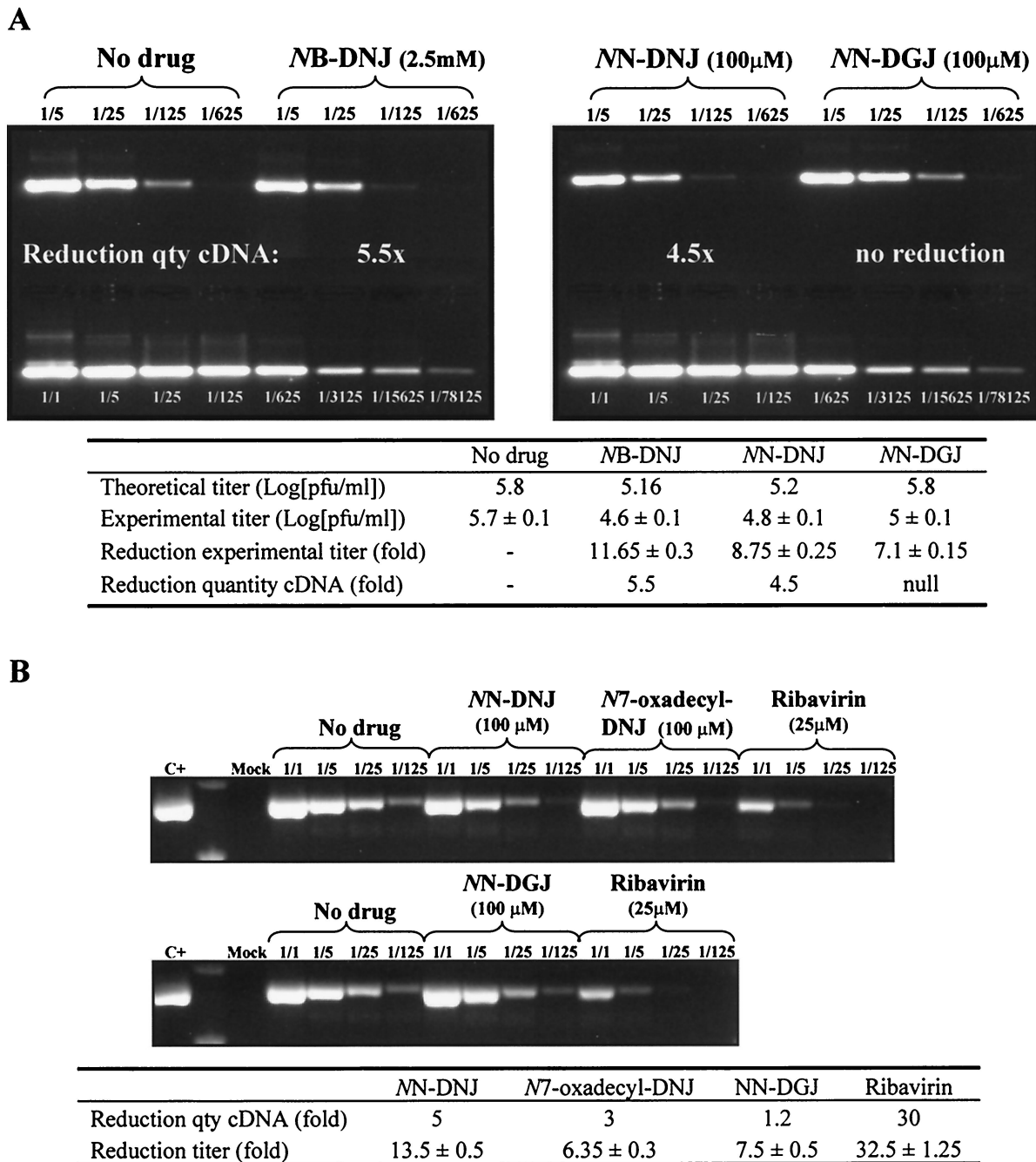


FIG. 5. Effect of iminosugar derivatives on viral secretion. MDBK cells were infected with either a cp (NADL) (A) or ncp (Pe515) (B) strain of BVDV at an MOI of 1 and grown for 24 h (A) or 48 h (B) in the absence or presence of different iminosugar derivatives at the concentrations indicated. Viral RNA was purified from the supernatant and used to perform a single-tube RT-PCR analysis. Then 10  $\mu$ l (A) or 20  $\mu$ l (B) of the RT-PCR product was loaded and run on a 2% agarose gel stained with ethidium bromide. (A) Result obtained using the cp NADL strain. The quantity of cDNA was determined by densitometry analysis. The experimental titer was measured by the plaque reduction assay, while the theoretical titer was calculated using the linear regression curve obtained with the standard curve (established from a virus stock with a titer of at  $2 \times 10^7$  PFU/ml). A summary of these results is presented in the table shown below the gels. (B) Result obtained using the ncp Pe515 strain. The quantity of cDNA was determined by densitometry analysis, and the experimental titer was measured by the plaque reduction assay. The values corresponding to the reduction in cDNA quantity and the reduction in the titer, obtained by comparison of treated and nontreated samples, are presented in the table below the gels.

to a certain extent. This is, however, not sufficient to account for all of the antiviral effect observed. However, the particles that do get secreted are not as infective as wild-type particles, which accounts for the stronger antiviral effect observed.

Long-alkyl-chain DGJ derivatives do not inhibit the secretion of viral RNA. Their antiviral effect is entirely due to a second mechanism, which reduces the infectivity of the particles released.

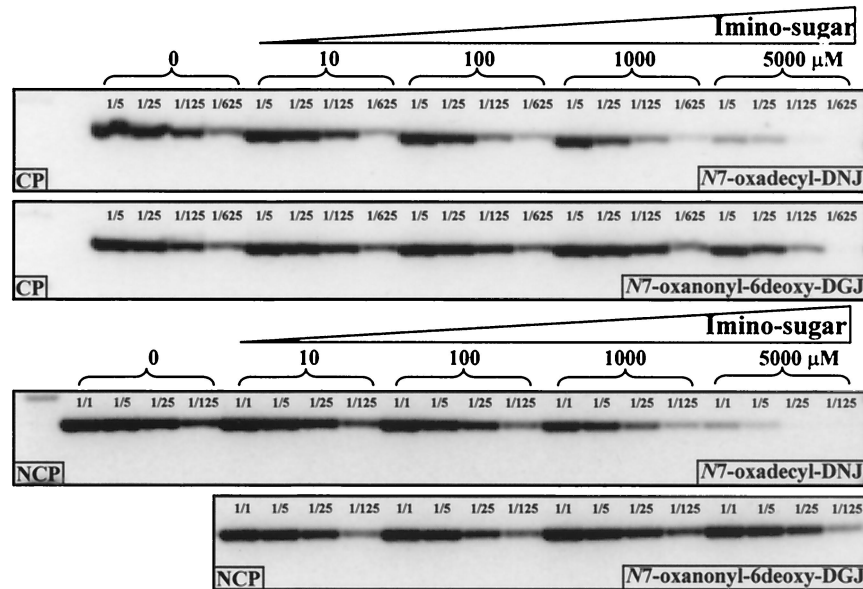


FIG. 6. Effect of iminosugar derivatives on viral secretion: a dose-response analysis. MDBK cells were infected with either a cp (NADL) or ncp (Pe515) strain of BVDV at an MOI of 1 and grown for 24 h (cp) or 48 h (ncp) in the absence or presence of increasing amounts of two different iminosugar derivatives. Viral RNA was purified from the supernatant and used to perform a single-tube RT-PCR analysis, as described in Materials and Methods. Then 10  $\mu$ l (cp) or 20  $\mu$ l (ncp) was loaded and run on an ethidium bromide-stained 2% agarose gel. Computerized-inverted images of the gels are presented. The type of virus and drug used are indicated in boxes within the frames of the gels.

**Iminosugar derivatives show the same antiviral effect against the cp and the ncp biotypes of BVDV.** BVDV strains fall into two biotypes which differ according to their pathogenicity in cultured cells. The ncp strains cause no visible in vitro pathogenicity, whilst the cp strains cause cell death by apoptosis (28). The cp virus was used for antiviral screening, since it induces plaques in the host cell monolayers, which can be easily quantified. However, the virological features of the ncp biotype may more closely resemble those of HCV, the ultimate proposed target for the drugs developed. Therefore, we repeated the RT-PCR experiment described above using the ncp Pe515 strain. Ribavirin, which inhibits viral RNA replication, was used as a control. Treatment with ribavirin led to a 30-fold decrease in the amount of viral RNA secreted from cells, which correlates with the 32-fold reduction in infectious viral titer (Fig. 5B). Mirroring the results obtained with the cp biotype, there was a discrepancy between the theoretical and experimentally determined viral titer of samples treated with DNJ and DGJ derivatives. The two DNJ derivatives tested, NN-DNJ and *N7*-oxadecyl-DNJ (chemical structures in Fig. 1), inhibited the secretion of viral particles, as measured by the reduction of viral RNA levels in the supernatants (table in Fig. 5B) 5- and 3-fold, respectively. This alone could not explain the 13.5- and 6.35-fold reduction in the amount of infectious virus. Treatment with NN-DGJ did not lead to a significant reduction (1.2-fold drop) of secreted viral RNA, but it did lead to a 7.5-fold reduction in infectious viral titer. The results obtained using the ncp biotype confirmed those achieved with the cp biotype. They indicate that part of the antiviral effect achieved with long-alkyl-chain DNJ derivatives and all of the antiviral effect achieved with long-alkyl-chain DGJ derivatives is due to the creation of virus particles with reduced infectivity.

**Long-alkyl-chain DNJ derivatives inhibit viral RNA secretion in a dose-dependent manner.** The RT-PCR experiments described above were performed at fixed inhibitor concentrations. To observe the dose-response effect, we chose iminosugar derivatives which combined good antiviral efficacy with low toxicity. *N7*-Oxadecyl-DNJ and *N7*-oxanonyl-6deoxy-DGJ, representing the two classes of iminosugar derivatives used in this study, have  $IC_{50[Moi 1]}$  of  $\sim 80$  and  $125 \mu$ M, respectively. The oxygen atom in the side chain results in a comparatively low in vitro toxicity, with  $CC_{50}$ s of  $\sim 5$  and  $4$  mM, respectively. The reduced toxicity allowed the use of higher inhibitor concentrations in the assay and the establishment of a dose-response curve. The experiment was performed using both the cp and ncp strain of BVDV. The virus-derived RT-PCR signal decreased with increasing concentrations of *N7*-oxadecyl-DNJ (Fig. 6), indicating an increasing inhibition of viral RNA secretion. With *N7*-oxanonyl-6deoxy-DGJ, only a slight reduction of viral RNA secretion was observed at the highest inhibitor concentration used (5 mM). However, since the  $CC_{50}$  of this compound lies between 4 and 5 mM, this reduction is most probably caused by general toxicity of the compound and not by a specific antiviral mechanism. These results show that long-alkyl-chain DNJ derivatives inhibit viral RNA secretion in a dose-dependent manner. They further show that while *N7*-oxanonyl-6deoxy-DGJ reduces the infectious viral titer, it does not reduce the amount of viral RNA secreted. This confirms the data obtained with NN-DGJ, showing that long-alkyl-chain DGJ derivatives exert their antiviral effect solely via the production of virus particles with reduced infectivity.

**DNJ and DGJ derivatives with long alkyl side chains change the pattern of envelope glycoprotein dimer formation in the ER of infected cells.** Our investigations into the cause of the



formation of less infectious particles focused on the viral envelope glycoproteins and their dimerization in the ER. We have shown previously that treatment with NB-DNJ causes misfolding of the envelope proteins (5). The antiviral effect of NB-DNJ correlated with the misfolding of BVDV envelope glycoproteins and the impairment of their association into E1-E2 heterodimers. For the shorter-alkyl-side-chain derivative NB-DNJ, this effect was entirely due to  $\alpha$ -glucosidase inhibition mediated by the iminosugar headgroup, since the galactose analogue with the same alkyl chain length (NB-DGJ) did not show any antiviral effect. In this study we investigated the extent to which the longer-alkyl-chain DNJ derivatives impair glycoprotein dimer formation in the ER. DGJ derivatives were used as a control. MDBK cells infected with ncp BVDV (MOI of 1) were treated with 100  $\mu$ M NN-DNJ, N7-oxadecyl-DNJ, NN-DGJ, or N7-oxanonyl-6deoxy-DGJ. At 48 h p.i., whole-cell lysates were prepared and analyzed under non-reducing conditions by SDS-PAGE followed by Western blotting and probing with MAb WB166, which recognizes a linear E2 epitope.

Figure 7A shows the accumulation of glycoprotein dimers formed in the ER in the presence of iminosugars. While no major change in the overall quantity of E1-E2 dimers was observed between nontreated (lane 2) and treated (lanes 3 to 6) samples, treated samples showed a large increase in the number of E2-E2 homodimers. This result shows that treatment with long-alkyl-chain iminosugar derivatives leads to a changed dimerization pattern of viral glycoproteins within the ER. The Western blot (Fig. 7A) revealed a moderate decrease in the quantity of E1-E2 detected in the presence of DNJ-based iminosugars (lanes 3 and 4). This slight reduction is probably due to a limited inhibition of the ER  $\alpha$ -glucosidases and subsequent misfolding and impairment of the association of E1 and E2 into heterodimers, as shown previously for NB-DNJ (5).

To confirm this observation, experiments were performed using both ncp and cp strains of BVDV, as well as increasing concentrations of N7-oxadecyl-DNJ and N7-oxanonyl-6deoxy-DGJ. At 48 h p.i., whole-cell lysates were prepared and analyzed under nonreducing conditions by SDS-PAGE followed by Western blotting and probing with MAb WB166 (Fig. 7B). The envelope glycoprotein dimers were quantified by a densitometric analysis. The value corresponding to the ratio of E2-E2 to E1-E2 was calculated for every sample and is shown at the bottom of each lane (with the ratio of E2-E2 to E1-E2 in the untreated sample being regarded as 100%). For both drugs, the quantity of E2-E2 in relation to the quantity of E1-E2 increased in a dose-dependent manner. The increase was greater with N7-oxadecyl-DNJ than with N7-oxanonyl-6deoxy-DGJ, which may be due to the concomitant slight reduction in the quantity of E1-E2, caused by DNJ-mediated ER  $\alpha$ -glucosidase inhibition. The upward shift observed in DNJ derivative-treated samples is a marker for this inhibition, which causes the glycoproteins to misfold and impairs their association into heterodimers. For N7-oxanonyl-6deoxy-DGJ, no such misfolding and reduction in the quantity of E1-E2 was observed, and the accumulation of E2-E2 homodimers correlated with the antiviral action of this compound.

We have established in this study that when attached to a shorter alkyl chain ( $C_4$ ), only the DNJ derivative shows anti-

viral activity whereas the DGJ-derivative has no effect. However, when linked to a longer alkyl chain ( $C_9$ ), both DNJ and DGJ derivatives show an antiviral effect in the in vitro BVDV system. The antiviral effect observed with, for example, NN-DGJ is therefore assumed to be mediated via the longer alkyl side chain. As shown in Fig. 5 and 6, this antiviral effect may be due to the creation of viral particles with reduced infectivity, which in turn may be caused by a change in the dimerization pattern of the viral envelope glycoproteins. In the next experiment, the correlation between the presence of a long alkyl side chain and the accumulation of E2-E2 dimers in the cells was assessed. MDBK cells infected with cp BVDV were treated with increasing amounts of two short-alkyl-chain (NB-DNJ or NB-DGJ) or two long-alkyl-chain iminosugar derivatives (NN-DNJ or NN-DGJ). At 24 h p.i., whole-cell lysates were analyzed by SDS-PAGE under nonreducing conditions followed by Western blotting and probing with MAb WB166 (Fig. 8). As expected, no major change in the quantity of E1-E2 and E2-E2 was observed with NB-DGJ. For NB-DNJ, while no significant change in the quantity of E2-E2 was detected, a lower mobility of both E1-E2 and E2-E2 dimers due to the presence of hyperglucosylated N-linked glycans was observed, as well as a reduction of the quantity of E1-E2 due to the impairment of their dimerization caused by misfolding of the envelope proteins. Thus, the antiviral effect of NB-DNJ correlated with the inhibition of the  $\alpha$ -glucosidases alone, as shown previously (5). For the longer-alkyl-side-chain derivative NN-DGJ, while no reduction in the quantity of E1-E2 was detected, a significant increase in the quantity of E2-E2 was observed. This abnormal accumulation of E2-E2 correlated with the antiviral effect of this compound. For NN-DNJ, an even greater accumulation of E2-E2 was observed. This accumulation was dose dependent and increased with increasing concentrations of NN-DNJ. At the same time, no significant reduction in the quantity of E1-E2 and only a very moderate upward shift of the bands were observed, indicating the lack of correlation between the antiviral activity of NN-DNJ and its ability to inhibit ER  $\alpha$ -glucosidases. However, the residual activity against  $\alpha$ -glucosidases, indicated by the slight shift, could explain why a greater accumulation of E2-E2 was observed with NN-DNJ in comparison to NN-DGJ.

**The envelope glycoprotein dimer composition of the virion changes on treatment with long-alkyl-chain iminosugar derivatives.** To determine whether the change in the ratio of E1-E2 to E2-E2 dimers synthesized within drug-treated cells was reflected in released virions, a Western blot analysis was performed with virus particles harvested directly from the cell culture medium. A total of  $4 \times 10^7$  MDBK cells were infected at an MOI of 1 and treated with different drugs (NB-DNJ at 2.5 mM and NN-DNJ and NN-DGJ at 150  $\mu$ M) or left untreated. After 24 h, the medium (90 ml in total) was harvested, centrifuged at  $5,000 \times g$  for 30 min to separate viral particles from cells, split in two, and processed by polyethylene glycol (PEG) 8000 precipitation or by centrifugation through a 20% sucrose cushion. Proteins from pellets were separated by SDS-PAGE under nonreducing conditions and subjected to Western blot analysis using an anti-E2 antibody (MAb WB166) (Fig. 9). When virus-infected cells were treated with NB-DNJ or NN-DNJ, the quantity of total virion-associated E2 released from cells was reduced, confirming the inhibition of secretion

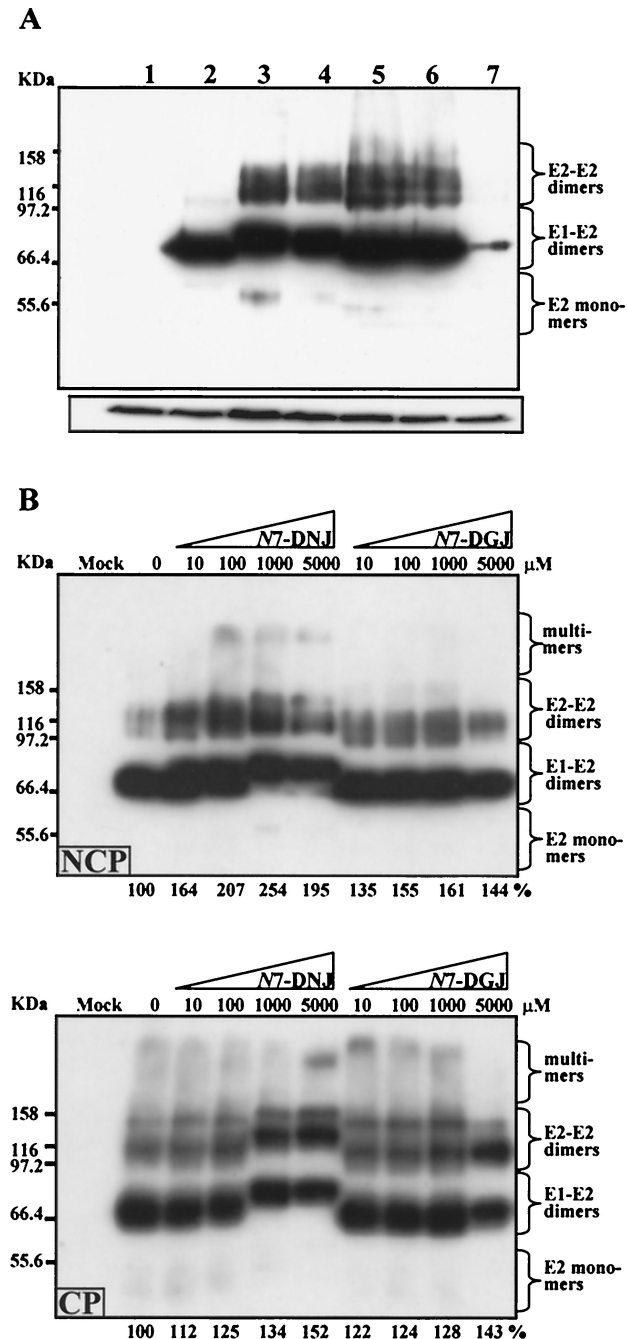


FIG. 7. Effect of iminosugar derivatives on the accumulation of E2-E2 homodimers and E1-E2 heterodimers in the ER. (A) MDBK cells were mock infected (lane 1) or infected with the ncp strain (Pe515) at an MOI of 1 and grown in the absence (lane 2) or presence of 100  $\mu$ M iminosugar derivatives (*NN-DNJ*, lane 3; *N7-oxadecyl-DNJ*, lane 4; *NN-DGJ*, lane 5; *N7-oxanonyl-6deoxy-DGJ*, lane 6). Ribavirin, which inhibits viral RNA replication, was used as a control (lane 7). At 48 h p.i., the cells were lysed and proteins were separated by SDS-PAGE (10% polyacrylamide) under nonreducing conditions. A Western blot analysis was performed using MAb WB166 (diluted 1:1,000), which recognizes the E2 glycoprotein regardless of its state of folding. In the bottom panel, the same blot was hybridized with an anti-actin antibody (loading control). (B) The same experiment as described for panel A was performed, but this time both cp and a ncp strains of BVDV were used for the infection. *N7-oxadecyl-DNJ* (abbreviated *N7-DNJ*) and *N7-oxanonyl-6deoxy-DGJ* (abbreviated *N7-DGJ*) were used at the concentrations indicated. The autoradiographs were quantified. The ratio

shown in Fig. 5A. This reduction was more pronounced with the short-alkyl-side-chain-containing *NB-DNJ* than with *NN-DNJ*, confirming the trend observed in the RT-PCR assay (Fig. 5). After treatment with *NN-DGJ*, a slight increase in the quantity of total E2 was detected. This correlated with the increased accumulation of E2 observed in cells and with the fact that *NN-DGJ* did not inhibit viral secretion. The relative quantity of E1-E2 and E2-E2 was determined by computer-assisted densitometry analysis. The percentage reflecting the increase of the quantity of E2-E2 with respect to the quantity of E1-E2 was calculated (table in Fig. 9). The results indicated that compared to untreated infected cells, proportionally more E2-E2 homodimers were present in virus particles released from drug-treated cells, suggesting that drug treatment induced a change of the envelope glycoprotein dimer composition of the virus particles. For *NN-DGJ*, which, unlike *NB-DNJ* and *NN-DNJ*, does not impair the secretion of viral particles (Fig. 5), the change in the viral envelope composition directly correlated with the creation of viral particles with reduced infectivity and could explain the drop in infectious titer. For *NB-DNJ* and *NN-DNJ*, the antiviral effect observed was due both to an inhibition of the secretion of virus particles and the production of particles with reduced infectivity. The latter correlated with the change in the glycoprotein dimer composition of the viral envelope.

## DISCUSSION

The use of iminosugars containing the glucose analogue DNJ as antiviral agents against different viruses has been suggested since the late 1980s (3, 4, 8–12, 16, 18, 19, 29). While the action of two of them, DNJ and *NB-DNJ*, has been extensively described in the literature, the discovery of the antiviral action of a longer-alkyl-chain derivative of DNJ, *NN-DNJ*, was reported only recently (29). In the first part of this report we presented antiviral and toxicity data for newly developed iminosugar derivatives. Several major discoveries were made during this phase of development and research, which was aimed at finding more potent and less toxic antivirals. While investigating whether the increased antiviral activity observed with *NN-DNJ* (compared to that of *NB-DNJ*) was due to an effect mediated by its long alkyl side chain, the  $C_9$  derivative of DGJ, *NN-DGJ*, was synthesized and found to be as effective as *NN-DNJ* in antiviral tests. This raised an interesting question about the mechanism of action of this molecule and also led us to doubt that the antiviral effect observed with *NN-DNJ* was indeed entirely due to the inhibition of the ER  $\alpha$ -glucosidases, as assumed previously. Because the shorter-alkyl-side-chain derivative *NB-DGJ* did not have any antiviral activity, it was hypothesized that the length of the alkyl side chain played an important role. However, nonylamine on its own did not show any specific antiviral activity, indicating that both the DGJ

of the amount of E2-E2 to the amount of E1-E2 was established, and the percentages, with the ratio of the amount of E2-E2 to the amount of E1-E2 without inhibitor present set at 100%, are shown at the bottom of each gel. Size standards are indicated on the left, and immunoreactive proteins and complexes are indicated on the right.

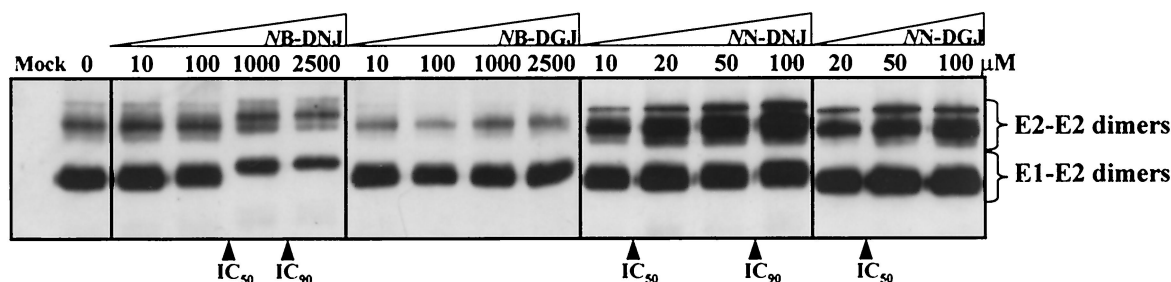


FIG. 8. Correlation between the presence of a long alkyl chain branched on DNJ or DGJ head groups and the abnormal accumulation of E2-E2 dimers. MDBK cells were mock infected or infected with the cp (NADL) strain of BVDV at an MOI of 1 and grown for 24 h in the absence or presence of iminosugar derivatives at the concentrations indicated. The cells were lysed, and proteins were separated by SDS-PAGE (10% polyacrylamide) under nonreducing conditions. A Western blot analysis was performed using MAb WB166 (diluted 1:1000). The  $IC_{50}$  and  $IC_{90}$  of each drug are indicated by arrows. No  $IC_{50}$  or  $IC_{90}$  is indicated for NB-DGJ, since this compound is not antiviral.

head group and the  $C_9$  alkylchain were responsible for the antiviral effect observed.

Efficacy and toxicity are the two most important aspects in the search for potential antivirals, with toxicity often being the limiting factor. Judged by their selectivity index ( $CC_{50}/IC_{50}$ ), the best iminosugar derivative of each class tested in the *in vitro* BVDV system were *N7-oxadecyl-DNJ* and *N7-oxanonyl-6deoxy-DGJ*. The latter has the added advantage of being only a very weak inhibitor of the ceramide-specific glucosyltransferase (Butters, unpublished), which would minimize side effects related to the inhibition of glycosphingolipid synthesis. Furthermore, the lowered toxicity, which is due to the oxygen atom at position 7 in the alkyl chain of both *N7-oxadecyl-DNJ* and *N7-oxanonyl-6deoxy-DGJ*, may lead to improved *in vivo* results.

While the antiviral mechanism of NB-DNJ has been extensively studied using both BVDV (5) and HIV (9–11) as models, the molecular details of the antiviral action of long-alkyl-chain derivatives of DNJ, such as NN-DNJ, and of the newly identified long-alkyl-chain derivatives of DGJ were unknown. Using the method described by Purchio et al. (24) to measure the synthesis of viral genomic RNA and using pulse-chase experiments to analyze protein synthesis and processing, we have demonstrated that long-alkyl-chain iminosugar derivatives do not interfere with either the BVDV RNA replication process or viral protein synthesis and processing. Our previous work suggested that DNJ derivatives inhibit the secretion of BVDV (29). After optimizing the experimental conditions, we revisited this hypothesis and measured the effect of iminosugar derivatives on viral secretion during a single round of high-MOI infection by quantitative RT-PCR. Our results show that DNJ-based derivatives reduce the secretion of RNA-containing virus particles, while no decrease of the level of enveloped viral RNA occurs with DGJ-based derivatives, emphasizing a fundamental difference in the mechanism of action of these two classes of molecules. Treatment with NB-DNJ impairs the proper folding of E1 and E2 glycoproteins and their association into heterodimers, which is a direct effect of the ER  $\alpha$ -glucosidase inhibition (5). Although to a lesser extent, the longer-alkyl-side-chain derivative NN-DNJ also inhibits the ER  $\alpha$ -glucosidases (Fig. 3, 4, and 7), and the impaired viral secretion observed with this inhibitor is likely to be caused by the same effect of impaired heterodimer formation as seen with NB-DNJ. However, after treatment with either NB-DNJ or NN-DNJ, a discrepancy became apparent between the re-

duction in the quantity of secreted viral RNA and the reduction in titer. Such a discrepancy was not observed when ribavirin, an inhibitor acting at the level of RNA replication, was used in control experiments. These results suggest that the inhibition of secretion observed with NB-DNJ and NN-DNJ does not account for the entire antiviral effect caused by these molecules. Here we provided evidence that the creation of viral particles with reduced infectivity contributes to the antiviral effect to different extents, depending on the alkyl chain length attached to the iminosugar headgroup. For longer-alkyl-

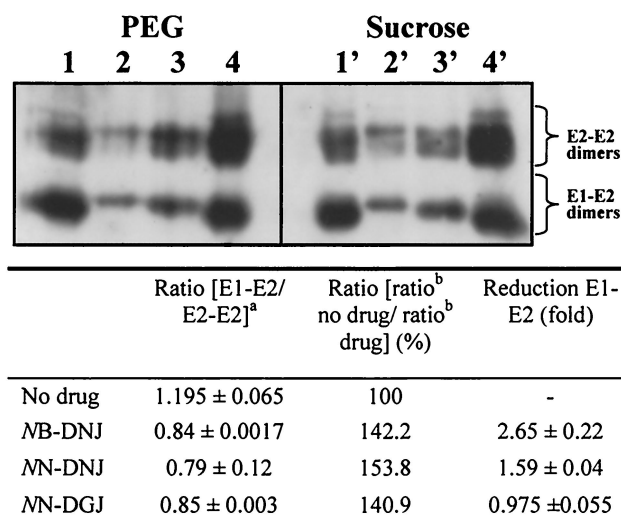


FIG. 9. Analysis of the envelope glycoprotein dimer composition of the virus particles released under drug treatment. MDBK cells were infected with the cp (NADL) strain of BVDV at an MOI of 1 and grown in the absence (lanes 1 and 1') or presence of NB-DNJ (2.5 mM; lanes 2 and 2'), NN-DNJ (150  $\mu$ M; lanes 3 and 3'), or NN-DGJ (150  $\mu$ M; lanes 4 and 4'). After 24 h, the medium was harvested, clarified at  $5,000 \times g$  for 30 min, split in two, and processed by PEG 8000 precipitation ( $10,000 \times g$ ) or by centrifugation ( $90,000 \times g$ ) through a 20% sucrose cushion. Proteins from pellets were separated by SDS-PAGE (10% polyacrylamide) under nonreducing conditions and subjected to a Western blot analysis using the anti-E2 MAb WB166. The bands detected were quantified by densitometry. The ratio of the amount of E1-E2 to the amount of E2-E2 was established for every drug concentration used, and the percentages, with the ratio of the amount of E2-E2 to the amount of E1-E2 without inhibitor present set at 100%, are shown in the table below the gel. a, values are combined quantities (determined by densitometry analysis) from both gels; b, values were taken from the previous column.



chain-DGJ-based derivatives, the entire antiviral effect is mediated via the production of less infectious viral particles. Most of the antiviral effect observed with *NN*-DNJ and even some of the antiviral effect observed with the shorter-alkyl-chain derivative *NB*-DNJ is also due to the creation of particles of reduced infectivity. A first clue to what may be the cause of this reduced infectivity came when we analyzed the glycoprotein dimer composition of secreted viral particles. We found that in comparison to virions released from untreated cells, the ratio between E1-E2 heterodimers and E2-E2 homodimers had changed. These results were confirmed by observations made with material derived from infected cells. The long-alkyl-chain compounds, regardless of whether they carry a DGJ or DNJ head group, induce an accumulation of E2-E2 homodimers (by an unknown mechanism). The short-alkyl-chain compound *NB*-DNJ does not induce such an accumulation. However, as an ER  $\alpha$ -glucosidase inhibitor, *NB*-DNJ causes a decrease in the E1-E2 heterodimer formation and, by reducing the number of E1-E2 dimers, also changes the ratio between E2-E2 and E1-E2 dimers. The long-alkyl-chain compound *NN*-DNJ is also an ER  $\alpha$ -glucosidase inhibitor and combines both effects. The E2-E2 accumulation caused by the presence of the long alkyl chain may contribute predominantly to its antiviral effect. However, the additional antiviral effect caused by the ER  $\alpha$ -glucosidase inhibition may be the reason why DNJ-based compounds consistently achieve better  $IC_{90}$ s. Whether the changed glycoprotein dimer composition in secreted viral particles only reflects or indeed accounts for the reduced infectivity of these particles will have to be established. In the absence of proper tools to investigate the behavior of the other two envelope glycoproteins, E1 and  $E^{TMS}$ , the overall picture of the antiviral mechanism of action is still far from complete.

In cells treated with long-alkyl-chain iminosugar derivatives (*NN*-DNJ and *NN*-DGJ), the overall quantity of E2 protein increases, which is most noticeable in E2-E2 homodimers. Since we have shown that neither viral RNA replication nor protein synthesis is modified by these iminosugars, a differential protein degradation rate may be responsible for this phenomenon. In this model, treatment with long-alkyl-chain iminosugar derivatives would lead to a delayed viral protein degradation. However, viral protein degradation may not be affected in cells treated with shorter-alkyl-chain iminosugar derivatives, which would explain the absence of overall accumulation of E2 protein in *NB*-DNJ treated cells. This hypothesis remains to be tested.

#### ACKNOWLEDGMENTS

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#### REFERENCES

- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1994. Current protocols in molecular biology. Greene and Wiley-Interscience, New York, N.Y.
- Baginski, S. G., D. C. Pevear, M. Seipel, S. C. Sun, C. A. Benetatos, S. K. Chunduru, C. M. Rice, and M. S. Collett. 2000. Mechanism of action of a pestivirus antiviral compound. *Proc. Natl. Acad. Sci. USA* **97**:7981-7986.
- Block, T. M., F. M. Platt, X. Lu, W. Gerlich, G. Foster, B. Blumberg, and R. A. Dwek. 1994. Secretion of human hepatitis B virus is inhibited by the imino sugar *N*-butyldeoxyjirimycin. *Proc. Natl. Acad. Sci. USA* **91**:2235-2239.
- Block, T. M., X. Lu, A. Mehta, B. Blumberg, B. Tennant, M. Ebling, B. Korba, D. M. Lansky, G. S. Jacob, and R. A. Dwek. 1998. Treatment of chronic hepatitis B virus infection in a woodchuck animal model with an inhibitor of protein folding and trafficking. *Nat. Med.* **4**:610-615.
- Branza-Nichita, N., D. Durantel, S. Carroué-Durantel, R. A. Dwek, and N. Zitzmann. 2001. Antiviral effect of *N*-butyldeoxyjirimycin against bovine viral diarrhoea virus correlates with misfolding of the E2 envelope proteins and impairment of their association into E1-E2 heterodimers. *J. Virol.* **75**:3527-3536.
- Butters, T. D., R. A. Dwek, and F. M. Platt. 2000. Inhibition of glycosphingolipid biosynthesis: application to lysosomal storage disorders. *Chem. Rev.* **100**:4683-4696.
- Butters, T. D., L. A. G. M. van den Broek, G. W. J. Fleet, T. M. Krulle, M. R. Wormald, R. A. Dwek, and F. M. Platt. 2000. Molecular requirements of imino sugars for the selective control of N-linked glycosylation and glycosphingolipid biosynthesis. *Tetrahedron Asymmetry* **11**:113-124.
- Courageot, M.-P., M.-P. Frenkiel, C. Duarte Dos Santos, V. Deubel, and P. Desprès. 2000. Alpha-glucosidase inhibitors reduce dengue virus production by affecting initial steps of virion morphogenesis in the endoplasmic reticulum. *J. Virol.* **74**:564-572.
- Fischer, P. B., M. Collin, G. B. Karlsson, W. James, T. D. Butters, S. J. Davis, S. Gordon, R. A. Dwek, and F. M. Platt. 1995. The  $\alpha$ -glucosidase inhibitor *N*-butyldeoxyjirimycin inhibits human immunodeficiency virus entry at the level of post CD4 binding. *J. Virol.* **69**:5791-5797.
- Fischer, P. B., G. B. Karlsson, T. D. Butters, R. A. Dwek, and F. M. Platt. 1996a. *N*-Butyldeoxyjirimycin mediated inhibition of human immunodeficiency virus entry correlates with changes in antibody recognition of the V1/V2 region of gp120. *J. Virol.* **70**:7143-7152.
- Fischer, P. B., G. B. Karlsson, T. D. Butters, R. A. Dwek, and F. M. Platt. 1996b. *N*-Butyldeoxyjirimycin mediated inhibition of human immunodeficiency virus entry correlates with impaired gp120 shedding and gp41 exposure. *J. Virol.* **70**:7153-7160.
- Fleet, G. W., A. Karpas, R. A. Dwek, L. E. Fellows, A. S. Tymes, S. Petursson, S. K. Nangoong, N. G. Ramsden, P. W. Smith, J. C. Son, et al. 1988. Inhibition of HIV replication by amino-sugar derivatives. *FEBS Lett.* **237**:128-132.
- Hammond, C., and A. Helenius. 1994. Folding of VSV-G protein: sequential interaction with BiP and calnexin. *Science* **266**:456-458.
- Hebert, D. N., B. Foellmer, and A. Helenius. 1995. Glucose trimming and reglycosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell* **81**:425-433.
- Horikoshi T., K. D. Danenberg, T. H. Stadlbauer, M. Volkenandt, L. C. Shea, K. Aigner, B. Gustavsson, L. Leichman, R. Frosing, M. Ray, et al. 1992. Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. *Cancer Res.* **52**:108-116.
- Karpas, A., G. W. Fleet, R. A. Dwek, S. Petursson, S. K. Nangoong, N. G. Ramsden, G. S. Jacob, and T. W. Rademacher. 1988. Aminosugar derivatives as potential anti-human immunodeficiency virus agents. *Proc. Natl. Acad. Sci. USA* **85**:9229-9233. (Erratum, **86**:5625, 1989.)
- Klausner, R. D., and R. Sitia. 1990. Protein degradation in the endoplasmic reticulum. *Cell* **62**:611-614.
- Lu, X., A. Mehta, M. Dadmarz, R. A. Dwek, B. S. Blumberg, and T. M. Block. 1997. Aberrant trafficking of hepatitis B virus glycoproteins in cells in which N-glycan processing is inhibited. *Proc. Natl. Acad. Sci. USA* **94**:2380-2385.
- Mehta, A., X. Lu, T. M. Block, B. S. Blumberg, and R. A. Dwek. 1997. Hepatitis B virus (HBV) envelope glycoproteins vary drastically in their sensitivity to glycan processing: evidence that alteration of a single N-linked glycosylation site can regulate HBV secretion. *Proc. Natl. Acad. Sci. USA* **94**:1822-1827.
- Mehta, A., N. Zitzmann, P. M. Rudd, T. M. Block, and R. A. Dwek. 1998.  $\alpha$ -glucosidase inhibitors as potential broad based anti-viral agents. *FEBS Lett.* **430**:17-22.
- Mellor, H. R., A. Adam, F. M. Platt, R. A. Dwek, and T. D. Butters. 2000. High-performance cation-exchange chromatography and pulsed amperometric detection for the separation, detection, and quantitation of N-alkylated imino sugars in biological samples. *Anal. Biochem.* **284**:136-142.
- Peterson, J. R., A. Ora, P. N. Van, and A. Helenius. 1995. Transient, lectin-like association of calreticulin with folding intermediates of cellular and viral glycoproteins. *Mol. Biol. Cell* **6**:1173-1184.
- Pocock, D. H., C. J. Howard, M. C. Clarke, and J. Brownlie. 1987. Variation in the intracellular polypeptide profiles from different isolates of bovine virus diarrhoea virus. *Arch. Virol.* **94**:43-53.
- Purchio, A. F., R. Larson, and M. S. Collett. 1983. Characterization of virus-specific RNA synthesized in bovine cells infected with bovine viral diarrhoea virus. *J. Virol.* **48**:320-324.
- Rice C. M. 1996. Flaviviridae: the viruses and their replication, p. 932-958. In B. N. Fields, D. M. Knipe, P. M. Howley, et al. (ed.), *Fields virology*, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
- Schmittgen, T. D., B. A. Zakrajsek, A. G. Mills, V. Gorn, M. J. Singer, and M. W. Reed. 2000. Quantitative reverse transcription-polymerase chain re-



- action to study mRNA decay: comparison of endpoint and real-time methods. *Anal. Biochem.* **285**:194–204.
27. **Sullivan, D. G., and R. K. Akkina.** 1995 A nested polymerase chain reaction assay to differentiate pestiviruses. *Virus Res.* **38**:231–239.
28. **Thiel, H. J., P. G. W. Plagemann, and V. Moennig.** 1996. Pestiviruses, p. 1059–1073. *In* B. N. Fields, D. M. Knipe, P. M. Howley, et al. (ed), *Fields virology*, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
29. **Zitzmann, N., A. S. Mehta, S. Carrouée, T. D. Butters, F. M. Platt, J. McCauley, B. S. Blumberg, R. A. Dwek, and T. M. Block.** 1999. Imino sugars inhibit the formation and secretion of bovine viral diarrhoea virus, a pestivirus model of hepatitis C virus: implications for the development of broad spectrum anti-hepatitis virus agents. *Proc. Natl. Acad. Sci. USA* **96**:11878–11882.