BARUCH BLUMBERG – THE OXFORD-ROMANIAN CONNECTION

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Baruch Samuel Blumberg lived from 28 July 1925 till 5 April 2011 (Fig. 1). He completed his D. Phil in Oxford between 1955–57 working with Sandy Ogston, FRS. Sandy's scientific bent was for "sensible solutions" and his scientific style had an enormous effect on Baruch. Sandy's philosophy went far beyond the laboratory, and is summed up in one of his addresses "*For science is more than the search for truth, more than a challenging game, more than a profession. It is a life that a diversity of people lead together, in the closest proximity, a school for social living. We are members one of another".* Throughout his scientific life this was true of Barry, as he was known to all. He returned to Trinity College in 1972, as a Visiting Fellow, when Sandy was its President.

Barry Blumberg won the Nobel Prize for medicine in 1976 for the discovery of the hepatitis B virus. He showed that it could cause liver cancer and then helped develop a powerful vaccine against hepatitis B. It is now estimated that as many as one billion people have been vaccinated and that at least 20 million lives have been saved (Fig. 2).

In 1983–4, Barry returned to Oxford as the George Eastman Professor at Balliol College, and in 1989–1994 he became the first American Master of the College. On arriving in Oxford in 1989, he approached me asking if he could become a member of the Oxford Glycobiology Institute. He liked the ideas which we were just developing that different glycoforms of a glycoprotein could have different functions. He saw this as creating diversity for glycoproteins and saw similarities to polymorphism in proteins which had always been one of his interests.

His presence in the Institute had a profound effect on catalysing my interests in viruses. Initially he came in for one day per week but that became more frequent as our friendship grew. In 1991 the Glycobiology Institute moved into its own

building, still part of the Biochemistry Department, and Baruch's integration into the research was so prominent that he had his own office and we started to think of research that would combine the virus and glyco areas.

The arrival of Professor Tim Block, from Jefferson University, and Anand Mehta to work with us both provided the impetus. We had been developing an iminosugar in the laboratory which had antiviral properties (Fig. 3). We had used it in an HIV trial with the pharmaceutical company G.D. Searle, and although there was some efficacy, there were significant side effects at the high doses needed. By chance we had discovered in the Institute that it was also an inhibitor of glycolipid production at much lower doses and this was later successfully developed by Oxford GlycoSciences (OGS) as a treatment for Gaucher disease. Barry also became a member of the scientific board of OGS and he tried to steer their interests to anti-viral therapies.

As Barry said in his autobiography, *"Professor Raymond Dwek asked me to join the Glycobiology Institute, of which he was the Founder and Director; thus began a friendship and collaboration that has lasted since then. We established a program to study the glycosylation of the surface antigen of HBV, a little understood subject. The project quickly turned clinical when Timothy Block, a colleague and friend from Philadelphia, came to work on the project during a sabbatical year. We studied the mechanism of the use of partially synthetic sugars to interfere with the glycosylation of the virus in the liver and* in vitro*. This appeared to hinder the intracellular assembly of the virus in addition to other mechanisms of action. The class of sugar therapies has possible application for the treatment of HBV, HCV, and other viruses that have a glycoprotein surface coating and has resulted in a whole new area of research*."

Indeed, when Tim Block (1993) showed, however that, this iminosugar drug could be used to prevent secretion of hepatitis B, the antiviral programme of the Institute was established and has become the main focus of the research programmes today.

At the same time, Stefana Petrescu from Romania was spending time as a visiting scientist in the Institute. She had made the surprising finding that when she added the iminosugar to B16 cells, which were black, they turned white. The molecular understanding of that was worked out by Stefana Petrescu and Norica Branza–Nichita and others, notably Andrei Petrescu from the Biochemistry Institute in Romania and Mark Wormald in Oxford. This was transformative for Glycobiology for it led to the idea that envelope glycoproteins on viruses were folded to attain their 3D structures by a pathway that could be inhibited by these iminosugars.

Nicole Zitzmann had joined the Institute at this time (1997) and started a programme on hepatitis C. Norica Branza- Nichita joined Nicole's programme as a

postdoctoral researcher. Barry was keen on this programme and identified Nicole as an outstanding scientist who had to be nurtured. Indeed he was her mentor, writing to me frequently about her progress and ensuring that she had adequate resources. He was instrumental in her being appointed Deputy Director of the Institute, shortly before he died. Interestingly, when his book on hepatitis was published in 2002, he wrote in Nicole's copy*: "It has been a great pleasure to follow your work on the hepatitis viruses. You will have a big impact on the control of these diseases. Your friend and colleague."*

In 2001 I met Martine Rothblatt, the CEO of United Therapeutics (UT). Both Baruch and I were impressed by this multitalented, creative and imaginative person. We all became close friends and Martine and UT strongly supported our work on iminosugars as antivirals at the Institute (Figs. 4, 5). Baruch eventually became the Chairman of the Scientific Board of UT and was much involved in planning the upcoming clinical trials on antivirals for patients who have hepC as well as co-infections of hepB and HIV. Martine Rothblatt and UT also supported some of the antiviral work in the Institute of Biochemistry at Bucharest being done by Norica Branza-Nichita (Fig. 6).

In Oxford I was involved in many activities with Baruch, from the Magdalene Science Park to helping to plan science in the Negev in Israel. Indeed, in 2002 his inscription in his book to me read "My dear friend. We have done many things together and will do more in the future." How true that was! He was my closest friend and was at the institute for 22 years. I enjoyed every moment in his presence. We never had enough time to finish all our conversations and yet we could pick up again whenever we met with hardly a break. I thought he was indestructible and we were planning more trips together. In 2010 we went to Bucharest together and he was honoured by the Romanian Academy with Foreign Membership. He was thrilled to go and also to show his support for the remarkable Institute of Biochemistry that Stefana Petrescu and colleagues had now established (Figs. 7, 8).

Perhaps our expression of affection for Barry is summed up in the words of the poet, Yates:

"*Think where man's glory most begins and ends,*
And say my glory was, I had such a friend".

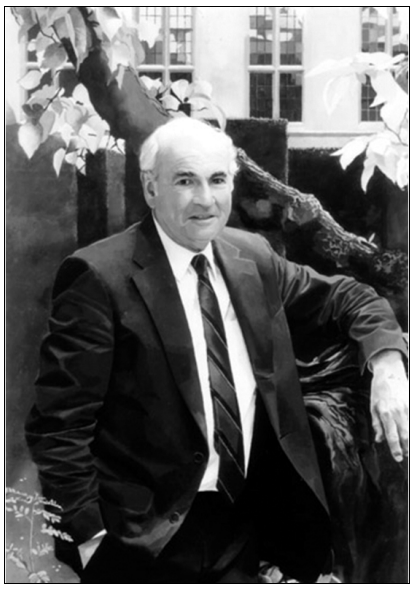

Fig. 1. – The official portrait of Baruch Blumberg in Balliol College.

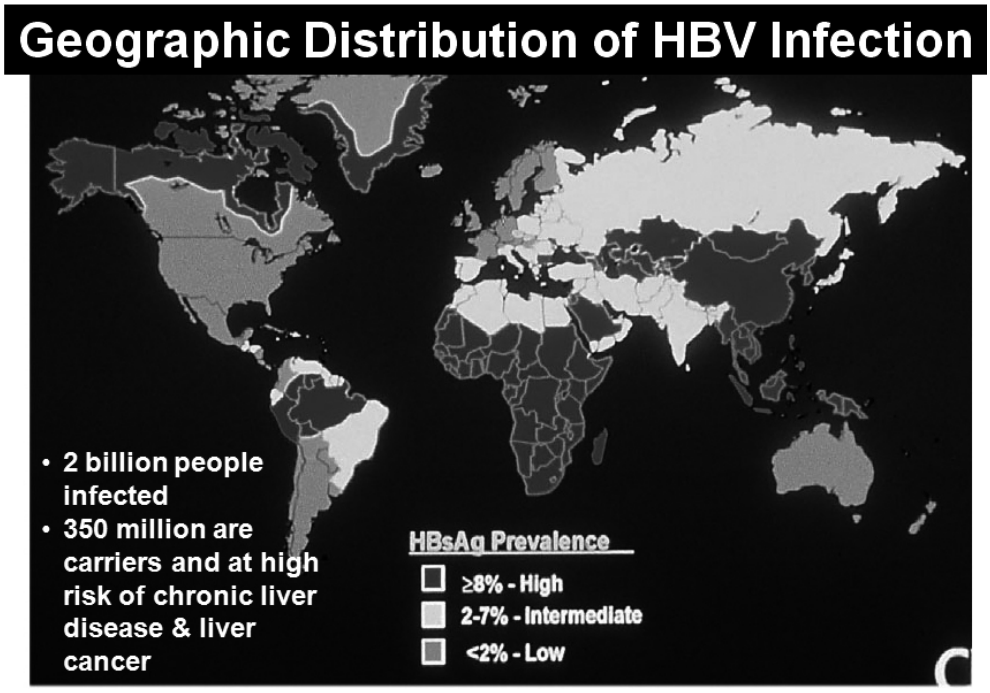

Fig. 2. – Prevalence of Hep B in the world,
and the number of people vaccinated and lives saved.

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Cell Biology

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

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Contributed by Baruch S. Blumberg, December 17, 1996

ABSTRACT **The role of N-linked glycosylation and glycan trimming in the function of glycoproteins remains a central question in biology. Hepatitis B virus specifies three glycoproteins (L, M, and S) that are derived from alternate translation of the same ORF. All three glycoproteins contain a common N-glycosylation site in the S domain while M possesses an additional N-glycosylation site at its amino terminus. In the presence of *N*-butyl-deoxnojirimycin (an inhibitor of α-glucosidase) virions and the M protein are surprisingly retained. Preliminary evidence suggests that the retained M protein is hyperglucosylated and localized to lysosomal vesicles. In contrast, the S and L proteins are secreted, and their glycosylation state is unaffected by the presence of the inhibitor. Site-directed mutagenesis provides evidence that virion secretion requires the glycosylation sequon in the pre-S2 domain of M. This highlights the potential role of the M protein oligosaccharide as a therapeutic target.**

Hepatitis B virus (HBV) is the human member of the *hepadnaviradae* family of viruses which infects over 300 million people worldwide. The HBV genome encodes for three related envelope proteins termed L, M, and S (Fig. 1*A*). The three envelope proteins are produced from a single ORF through alternative translation start sites. All three proteins have a common N-linked glycosylation site at position 146 of the S domain (Fig. 1*A*), while the M protein alone contains an additional site at position 4 of the pre-S2 domain (1).

A peculiar feature of these envelope glycoproteins is that they are secreted in the form of small, noninfectious, non-DNA-containing, lipoprotein particles. These subviral particles are secreted in vast numbers, often outnumbering the infectious HBV virion by 100,000:1 (1). Subviral particles are found in two forms: spheres, which consists mainly of the S and M proteins; and filaments, which contain a greater amount of the L protein (2).

All three envelope glycoproteins are important in the viral life cycle. While it has been shown that the L and S proteins are necessary for virion secretion (3), the role of M is in doubt (3, 4). The current model of virion formation involves DNA containing nucleocapsids budding into the lumen of the endoplasmic reticulum (ER) and secretion through the trans-Golgi network (5).

The N-linked glycosylation pathway is well defined, consisting of over 13 enzymes that are involved in processing within the ER and the Golgi apparatus (6). Specific inhibitors of this pathway can be used to probe the importance of N-linked glycosylation. The role of N-linked glycosylation in HBV has yet to be determined. Several reports have provided evidence that the N-linked glycans are not necessary for the secretion of the subviral particle (7, 8). In contrast, the secretion of virus requires both N-linked glycosylation (9) and N-linked glycan processing (10). However, it was unclear whether this resulted from a general effect of glycosylation or from a specific effect on a particular viral glycoprotein. To this end, we have now analyzed the glycosylation state and the secretion levels of the three envelope glycoproteins (S, M, and L) in the presence of the α-glucosidase inhibitor *N*-butyl-deoxnojirimycin (NB-DNJ). The α-glucosidases are the first enzymes involved in the glycan processing pathway and remove the terminal three glucose residues from the $Glc_3Man_9GlcNAc_2$ glycoform after it has been transferred from the dolichol diphosphate to the growing polypeptide backbone (reviewed in ref. 6). Site-directed mutagenesis on the individual glycan sites of the three glycoproteins has allowed more detailed conclusions as to which glycosylation site is important in viral secretion. The data presented in this paper, together with our previous work with tunicamycin (9), lead to the conclusion that the Asn-4 glycan site in the pre-S2 domain of M plays an important role in the secretion of HBV.

METHODS

Cells and Transfections. HepG2 cells were grown in RPMI 1640 medium (GIBCO/BRL) containing 10% fetal bovine serum (GIBCO/BRL). HepG2.2.15 cells were kindly provided by George Acs (Mt. Sinai Medical College, New York) and maintained as HepG2 cells but with the addition of 200 μg/ml of G418 (GIBCO/BRL). DNA transfection of HepG2 cells were performed as in ref. 3.

Detection of Intracellular Viral DNA. HepG2.2.15 cells were either left untreated or treated with 1000 μg/ml of the α-glucosidase inhibitor NB-DNJ (provided by Monsanto/Searle) for the indicated times and the total DNA extracted as described (11). DNA (25 μg) was digested with *Hin*dIII, resolved through a 1.2% agrose gel, and transferred to nylon membranes (Micron Separations, Westboro, MA). Membranes were then hybridized with a ^{32}P-labeled probe containing the total HBV genome and developed as described (11). The relaxed circular, linear, and closed circular DNA were confirmed by enzymatic digestion (data not shown).

Detection of HBV Proteins by ELISA. After 6 days, culture medium from HepG2.2.15 cells left uninhibited or inhibited with the 1000 μg/ml NB-DNJ were resolved through a discontinuous sucrose gradient as described (10). Fractions were collected and subjected to antigen detection by the ELISA method as described (10).

Release of Glycans and Labeling of Sugars. Subviral particles from untreated or NB-DNJ-treated cells were purified by double CsCl ultracentrifugation (2) and concentrated using an

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Abbreviations: HBV, hepatitis B virus; ER, endoplasmic reticulum; NB-DNJ, *N*-butyl-deoxnojirimycin.

Fig. 3. – The first paper showing the importance of glycosylation in hepatitis B and the use of an iminosugar.

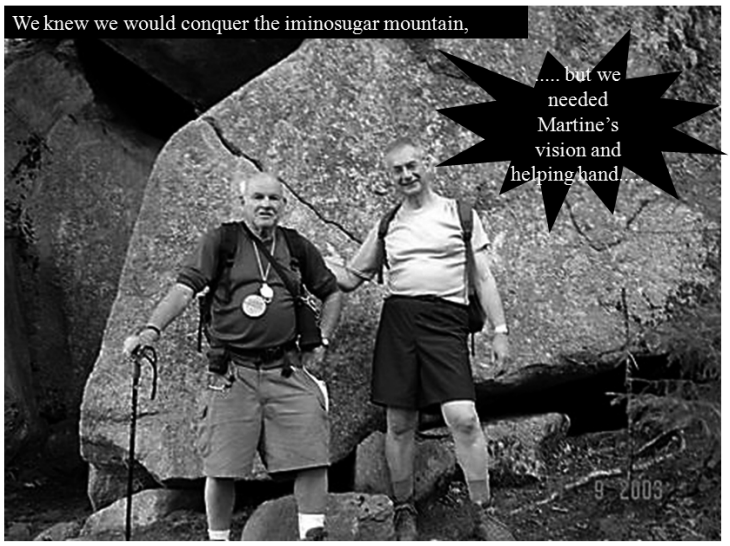


Fig. 4. – On the Appalachian way discussing iminosugars and United Therapeutics.

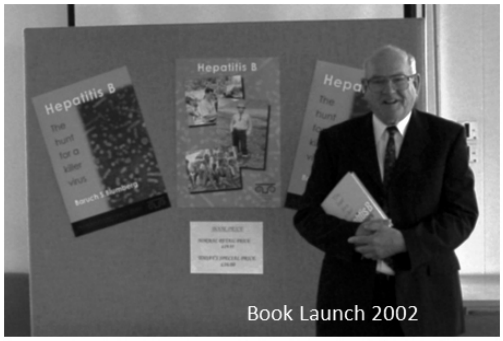
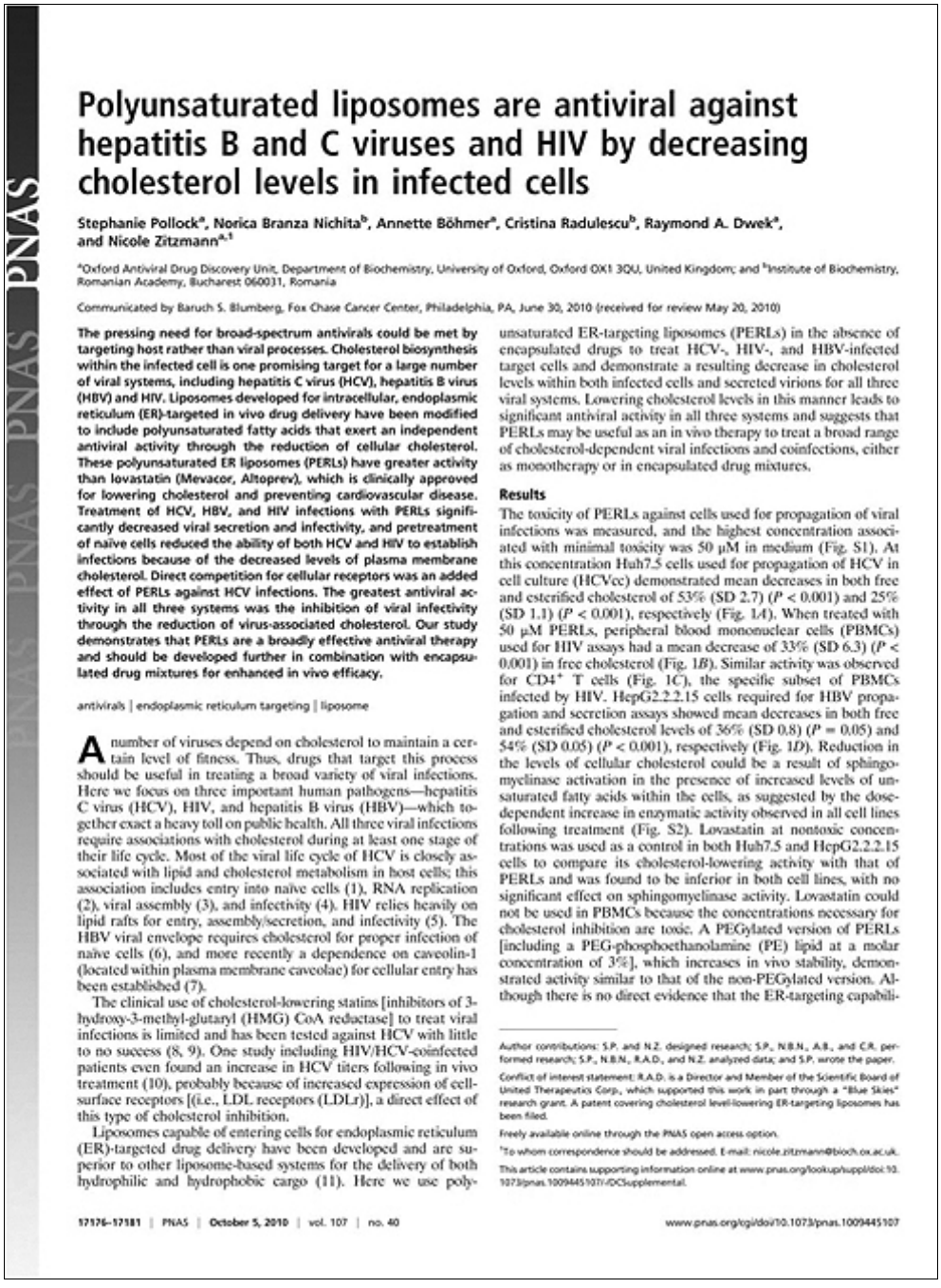


Fig. 5. – Barry in Oxford, at the Glycobiology Institute, at the launch of his book, at the opening (with Martine Rothblatt) of the United Therapeutics antiviral unit, directed by Nicole Zitzmann, and giving the first distinguished Unither Virology Lecture.



Polyunsaturated liposomes are antiviral against hepatitis B and C viruses and HIV by decreasing cholesterol levels in infected cells

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The pressing need for broad-spectrum antivirals could be met by targeting host rather than viral processes. Cholesterol biosynthesis within the infected cell is one promising target for a large number of viral systems, including hepatitis C virus (HCV), hepatitis B virus (HBV) and HIV. Liposomes developed for intracellular, endoplasmic reticulum (ER)-targeted in vivo drug delivery have been modified to include polyunsaturated fatty acids that exert an independent antiviral activity through the reduction of cellular cholesterol. These polyunsaturated ER liposomes (PERLs) have greater activity than lovastatin (Mevacor, Altoprev), which is clinically approved for lowering cholesterol and preventing cardiovascular disease. Treatment of HCV, HBV, and HIV infections with PERLs significantly decreased viral secretion and infectivity, and pretreatment of naïve cells reduced the ability of both HCV and HIV to establish infections because of the decreased levels of plasma membrane cholesterol. Direct competition for cellular receptors was an added effect of PERLs against HCV infections. The greatest antiviral activity in all three systems was the inhibition of viral infectivity through the reduction of virus-associated cholesterol. Our study demonstrates that PERLs are a broadly effective antiviral therapy and should be developed further in combination with encapsulated drug mixtures for enhanced in vivo efficacy.

antivirals | endoplasmic reticulum targeting | liposome

A number of viruses depend on cholesterol to maintain a certain level of fitness. Thus, drugs that target this process should be useful in treating a broad variety of viral infections. Here we focus on three important human pathogens—hepatitis C virus (HCV), HIV, and hepatitis B virus (HBV)—which together exact a heavy toll on public health. All three viral infections require associations with cholesterol during at least one stage of their life cycle. Most of the viral life cycle of HCV is closely associated with lipid and cholesterol metabolism in host cells; this association includes entry into naïve cells (1), RNA replication (2), viral assembly (3), and infectivity (4). HIV relies heavily on lipid rafts for entry, assembly/secretion, and infectivity (5). The HBV viral envelope requires cholesterol for proper infection of naïve cells (6), and more recently a dependence on caveolin-1 (located within plasma membrane caveolae) for cellular entry has been established (7).

The clinical use of cholesterol-lowering statins [inhibitors of 3-hydroxy-3-methyl-glutaryl (HMG) CoA reductase] to treat viral infections is limited and has been tested against HCV with little to no success (8, 9). One study including HIV/HCV-coinfected patients even found an increase in HCV titers following in vivo treatment (10), probably because of increased expression of cell-surface receptors [(i.e., LDL receptors (LDLr)], a direct effect of this type of cholesterol inhibition.

Liposomes capable of entering cells for endoplasmic reticulum (ER)-targeted drug delivery have been developed and are superior to other liposome-based systems for the delivery of both hydrophilic and hydrophobic cargo (11). Here we use polyunsaturated ER-targeting liposomes (PERLs) in the absence of encapsulated drugs to treat HCV-, HIV-, and HBV-infected target cells and demonstrate a resulting decrease in cholesterol levels within both infected cells and secreted virions for all three viral systems. Lowering cholesterol levels in this manner leads to significant antiviral activity in all three systems and suggests that PERLs may be useful as an in vivo therapy to treat a broad range of cholesterol-dependent viral infections and coinfections, either as monotherapy or in encapsulated drug mixtures.

Results

The toxicity of PERLs against cells used for propagation of viral infections was measured, and the highest concentration associated with minimal toxicity was 50 μM in medium (Fig. S1). At this concentration Huh7.5 cells used for propagation of HCV in cell culture (HCVcc) demonstrated mean decreases in both free and esterified cholesterol of 53% (SD 2.7) ($P < 0.001$) and 25% (SD 1.1) ($P < 0.001$), respectively (Fig. 1*A*). When treated with 50 μM PERLs, peripheral blood mononuclear cells (PBMCs) used for HIV assays had a mean decrease of 33% (SD 6.3) ($P < 0.001$) in free cholesterol (Fig. 1*B*). Similar activity was observed for $CD4^+$ T cells (Fig. 1*C*), the specific subset of PBMCs infected by HIV. HepG2.2.2.15 cells required for HBV propagation and secretion assays showed mean decreases in both free and esterified cholesterol levels of 36% (SD 0.8) ($P = 0.05$) and 54% (SD 0.05) ($P < 0.001$), respectively (Fig. 1*D*). Reduction in the levels of cellular cholesterol could be a result of sphingomyelinase activation in the presence of increased levels of unsaturated fatty acids within the cells, as suggested by the dose-dependent increase in enzymatic activity observed in all cell lines following treatment (Fig. S2). Lovastatin at nontoxic concentrations was used as a control in both Huh7.5 and HepG2.2.2.15 cells to compare its cholesterol-lowering activity with that of PERLs and was found to be inferior in both cell lines, with no significant effect on sphingomyelinase activity. Lovastatin could not be used in PBMCs because the concentrations necessary for cholesterol inhibition are toxic. A PEGylated version of PERLs [including a PEG-phosphoethanolamine (PE) lipid at a molar concentration of 3%], which increases in vivo stability, demonstrated activity similar to that of the non-PEGylated version. Although there is no direct evidence that the ER-targeting capabili-

Author contributions: S.P. and N.Z. designed research; S.P., N.B.N., A.B., and C.R. performed research; S.P., N.B.N., R.A.D., and N.Z. analyzed data; and S.P. wrote the paper.

Conflict of interest statement: R.A.D. is a Director and Member of the Scientific Board of United Therapeutics Corp., which supported this work in part through a "Blue Skies" research grant. A patent covering cholesterol level-lowering ER-targeting liposomes has been filed.

Freely available online through the PNAS open access option.

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Fig. 6. – The paper on the use of liposomes as antivirals submitted to PNAS by Baruch, reporting original results obtained in collaboration with Dr. Norica Nichita's group.

Fig. 7. – At the House of Parliament.
In the picture are also Stefana Petrescu, Norica Branza-Nichita, Richard Lerner and Raymond Dwek.

Fig. 8. – Baruch at the Institute of Biochemistry in Bucharest (Director Stefana Petrescu).
Also in the picture are Richard Lerner, President of Scripps USA and Raymond Dwek
from Oxford University.