and exposed to film at −70°C. β-actin mRNA was used as an internal standard.
14. P. Brown, *Antiviral Chem. Chemother.* **1**, 75 (1990); ——— in *Antiviral Agents: The Development and Assessment of Antiviral Chemotherapy*, H. J. Field, Ed. (CRC Press, Boca Raton, FL, 1988), vol. 2, pp. 13–28.
15. B. Ehlers and H. Diringer, *J. Gen. Virol.* **65**, 1325 (1984); C. F. Farquhar and A. G. Dickinson, *ibid.* **67**, 463 (1986); R. H. Kimberlin and C. A. Walker, *Antimicrob. Agents Chemother.* **30**, 409 (1986); M. Pocchiari, S. Schmittinger, C. Masullo, *J. Gen. Virol.* **68**, 219 (1987); Y. G. Xi, L. Ingrosso, A. Ladogana, C. Masullo, M. Pocchiari, *Nature* **356**, 598 (1992); R. H. Kimberlin and C. A. Walker, *Arch. Virol.* **78**, 9 (1993); K. T. Adjou *et al.*, *Antimicrob. Agents Chemother.* **39**, 2810 (1995).
16. F. Fomelli, R. Carsana, C. Pollini, *Cancer Res.* **47**, 5401 (1987).
17. F. Tagliavini *et al.*, *Cell* **79**, 695 (1994).
18. J. Sambrook, E. F. Fritsch, T. Maniatis, in *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).
19. We thank R. J. Kascsak for providing the antibody 3F4, L. Frigerio for preparation of histological sections, and M. Rochetti for statistical advice. Partially supported by the Italian Ministry of Health, Department of Social Services, Telethon-Italy (grant E.250) and the Consiglio Nazionale delle Ricerche (grant ST/74).

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A Membrane Network for Nutrient Import in Red Cells Infected with the Malaria Parasite

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The human malaria parasite *Plasmodium falciparum* exports an interconnected network of tubovesicular membranes (the TVM) that extends from the parasite's vacuolar membrane to the periphery of the red cell. Here it is shown that extracellular solutes such as Lucifer yellow enter the TVM and are delivered to the parasite. Blocking the assembly of the network blocked the delivery of exogenous Lucifer yellow, nucleosides, and amino acids to the parasite without inhibiting secretion of plasmodial proteins. These data suggest that the TVM is a transport network that allows nutrients efficient access to the parasite and could be used to deliver antimalarial drugs directly into the parasite.

Malaria afflicts 200 million to 300 million people and kills over 1 million children each year. The most fatal form of the disease is caused by the protozoan parasite *Plasmodium falciparum*. During its asexual cycle the parasite invades and develops in a vacuole in the mature erythrocyte, a cell devoid of all intracellular organelles and incapable of de novo protein synthesis and membrane turnover (*1*, *2*). In order to survive, the parasite exports antigens and imports extracellular nutrients (*3*–*5*) across the intraerythrocytic space by mechanisms that are largely unknown. Within 33 hours of infection the parasite induces the formation of the TVM that emerges from the parasitophorous vacuolar membrane and extends out to the erythrocyte membrane (*6*, *7*), suggesting that it may be a transport organelle.

TVM assembly can be blocked by inhibition of a resident parasite sphingomyelin synthase (called the sensitive sphingomyelin synthase or SSS) by *dl*-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP; Fig. 1) (*8*). Normal cells have shown a highly tubular network with a few large loops (Fig. 1A). PPMP-treated cells (Fig. 1B) were found to contain vesicles or rodlike elements, associated with the red cell membrane, free in the cytoplasm or in close association with the parasite. Few of the intraerythrocytic structures appeared to be interconnected, suggesting that the network is either highly constricted in regions that do not stain with Bodipy-ceramide, or it is fragmented.

To determine whether the state of TVM organization influences the transport properties of infected red cells, we incubated control (untreated) and PPMP-treated cells with the fluorescent solute Lucifer yellow (LY), which enters infected (but not uninfected) red cells by a parasite-induced, energy-dependent process (*9*). In mammalian cells LY is a marker for fluid phase pinocytosis; however, there is no endocytic activity at the infected red cell membrane (*10*, *11*). In a normal infected red cell, LY was detected in interconnected tubules and vesicles of the TVM and within the parasite (Fig. 1C). Labeled network structures extended along the periphery of the red cell across the intraerythrocytic space and back to the parasite. PPMP-treated cells appeared to be blocked in the intraerythrocytic delivery and accumulation of LY (Fig. 1D). Removal of PPMP restored TVM development (*8*) and the levels of LY transport and accumulation seen in untreated cells (*12*). PPMP does not block the channel or transporter for LY at the infected red cell membrane (*12*). Together, these data suggest that LY is delivered by the TVM to the parasite.

We next investigated whether other charged, low molecular mass solutes such as nucleosides, amino acids, and their toxic derivatives are also transported by the TVM by comparing parasite-induced accumulation of radiolabeled analogs in PPMP-treated and untreated cells. An important extracellular solute for parasite growth is the purine nucleoside adenosine. The parasite needs purines for growth but cannot synthesize them de novo. The uninfected erythrocyte has an endogenous nucleoside transporter that can be blocked by derivatives of 6-thiopurine nucleosides such as nitrobenzyl thioinosine (NBMPR); however, these compounds do not block parasite-induced nucleoside accumula-

Table 1. Inhibition of parasite-induced solute accumulation and use in infected red cells. Infected erythrocytes (at 10 to 20% parasitemia) treated with 5 μM PPMP for 36 hours were washed free of serum and incubated with the indicated radiolabeled substrates (*14*). The accumulation of [^{3}H]adenosine or [^{3}H]thymidine was measured over 30 s at room temperature. NBMPR (20 μM) was used to inhibit the endogenous red cell nucleoside transporter. Control cells were not treated with PPMP. Where indicated, 0.01% saponin was added. The accumulation of [^{3}H]orotic acid and [^{3}H]glutamate was measured over 30 min at 37°C. PPMP treatment had no effect on the uptake of any radiolabeled compound into uninfected red cells. The incorporation of [^{3}H]adenosine and [^{3}H]orotic acid was measured over 24 hours, after which the cells were harvested on glass fiber filters and the counts determined (*8*, *14*). These incorporation assays were carried out in the absence of NBMPR. The incorporation of [^{3}H]glutamate over 24 hours at 37°C was determined by measuring the acid-insoluble radioactivity precipitated on 3 MM Whatman filters (*17*, *14*). n.a., not applicable.

Substrate	Solute accumulation (10^{-5} nmol per 5×10^7 parasites)		Inhibition of accumulation (%)	Inhibition of incorporation (%)
	−PPMP	+PPMP		
[^{3}H]Adenosine	7.8	3.1	60 ± 20	97 ± 2
[^{3}H]Adenosine + saponin	6.2	5.6	9 ± 4	3 ± 2
[^{3}H]Orotic acid	17	1.5	91 ± 13	98 ± 1
[^{3}H]Thymidine	46	10	78 ± 6	n.a.
[^{3}H]Glutamate	14	3.6	74 ± 20	91 ± 5

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tion in infected erythrocytes (*13*). In contrast to NBMPR, PPMP substantially lowered the levels of parasite-induced [^{3}H]adenosine accumulation in infected red cells (~60% reduction within 30 s; Table 1). Incorporation of radiolabel into nucleic acids was also reduced by over 90% (Table 1).

To separate experimentally the intraerythrocytic transport of adenosine from its use by the parasite, we added saponin to both the control and PPMP-treated cells and then compared adenosine accumulation and incorporation in the continued absence or presence of PPMP (*14*). Saponin permeabilizes the red cell and intraerythrocytic TVM membranes but leaves the parasite intact and metabolically active (*15*). The level of adenosine accumulation in saponin-treated cells was ~80% that in normal cells (Table 1), confirming that most of the exogenous adenosine was rapidly delivered to the parasite. PPMP treatment had virtually no effect on the accumulation or incorporation of adenosine in the presence of saponin, indicating that PPMP does not affect adenosine metabolism in the parasite. It does not inhibit the uptake of adenosine at the parasite's surface or adenosine transporter at the surface of the infected red cell (*8*). These data suggest that PPMP acts by disrupting the TVM pathway that delivers adenosine to the parasite; this pathway functions at physiological concentrations of adenosine in plasma (less than 1 μM), suggesting that it may be used both in vivo as well as in vitro.

Parasite-infected red cells also take up the pyrimidine nucleosides thymidine and orotic acid from the extracellular medium (*16*). In PPMP-treated cells, the accumulation of orotic acid and thymidine was inhibited by 91 and 78%, respectively, and the incorporation of orotic acid into nucleic acids was reduced by 98% (Table 1). Because thymidine is not incorporated by plasmodial parasites, PPMP must exert its effect on transport rather than on metabolism. Because PPMP does not block the machinery for nucleic acid synthesis, these cells were not compromised in the ability to use orotic acid but rather in the transport of exogenous pyrimidines. As shown for purines, PPMP did not inhibit parasite pyrimidine transporters in the infected red cell membrane (*8*).

To determine whether amino acids are delivered by the TVM pathway, we examined the accumulation of L-glutamate, a compound that is efficiently internalized by infected but not uninfected red cells (*16*). The accumulation of glutamate and its incorporation into protein was greatly reduced in PPMP-treated infected erythrocytes (Table 1). Yet, as shown by immunolocalization studies (Fig. 2, A to E), these cells synthesized and exported numerous parasite-encoded polypeptides such as PfEMP2, PfHRP2, and Maurer's clefts, knobs, and intraerythrocytic loops and their resident marker proteins to the membrane and cytosol of the red cell. When the parasites were released from their host cell and TVM membranes and incubated with [^{35}S]Tran label, the levels of protein synthesis and export detected in the continued presence of PPMP were comparable (±10%) with those seen in control parasites incubated without PPMP (Fig. 2, F and G). In contrast, brefeldin blocked the export of several proteins (Fig. 2G) but did not diminish protein synthesis by the parasite (Fig. 2F). Low levels of brefeldin-insensitive export were also observed (*17*). Thus, PPMP-treated cells are not impaired in amino acid uptake at the parasite's surface, the machinery for protein synthesis, or protein

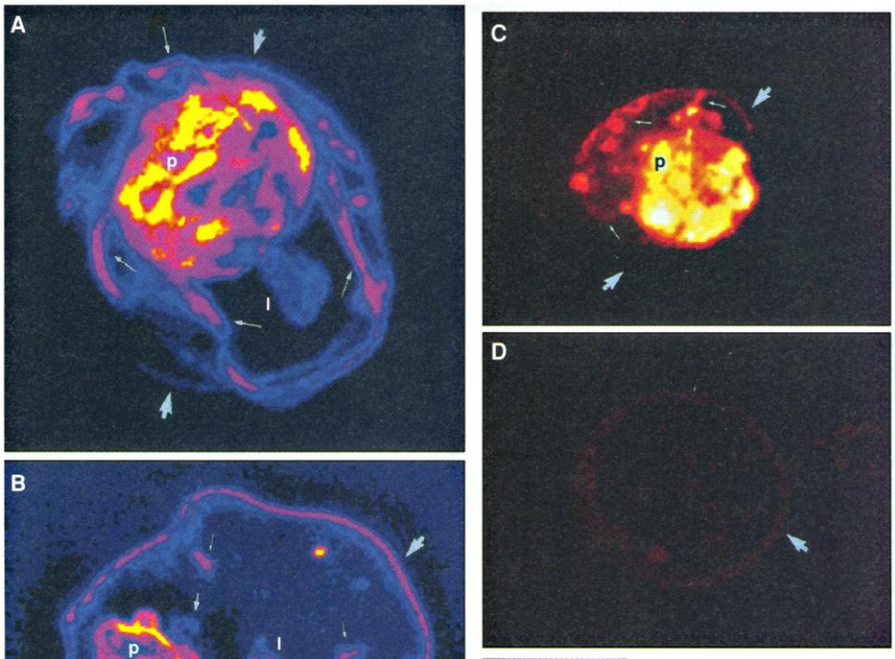

Fig. 1. Transport of Lucifer yellow (LY) by the TVM in *P. falciparum*–infected red cells and its disruption by PPMP. Pseudocolor projections of the TVM in normal (**A**) and PPMP-treated (**B**) infected red cells labeled with Bodipy-ceramide, which stains all intracellular membranes (*8*). Large arrowheads indicate periphery of the red cell; p, parasite; l, large vesicle or loop. In (A), long thin arrows show interconnected tubules of a normal TVM. In (B) (arrested TVM), short thin arrows indicate isolated rods; the short thick arrows indicate vesicular structures attached to the parasite; and x indicates TVM elements attached to the red cell membrane. Projections of the distribution of LY in normal (**C**) and PPMP-treated (**D**) infected red cells incubated at 1% hematocrit in RPMI 1640 medium containing LY (1 to 4 mg/ml) for 30 min at 37°C (*9*). Excess dye was removed by washing in RPMI 1640. Large arrowheads indicate the periphery of the red cell, and short thin arrows show LY staining in the TVM. All samples were viewed in a DeltaVision microscope and work station (Applied Precision, Seattle, WA). Twenty 200-nm optical sections taken through the depth of the cell were deconvoluted as described in (*24*) and reconstructed into a high-resolution, 0° projection. Scale bars beneath the images, 5 μm.

Table 2. Parasite-induced accumulation of 5-FO in TVM-arrested cells. Accumulation of 5-FO was determined as described for orotic acid (*19*) except that, because of its lower specific activity (0.05 Ci/mmol), the final extracellular concentration of [^{14}C]5-FO was 2.2 μM. Determination of the IC_{50} of 5-FO in TVM-arrested cells by [^{3}H]hypoxanthine incorporation ([^{3}H]Hx incorp.) and Giemsa staining was carried out with 12 hour–infected red cells treated with various concentrations of PPMP (0 to 3.3×10^{-6} M) and 5-FO (0 to 1×10^{-5} M) for 24 hours at 37°C (*19*). The IC_{50} was determined by plotting parasitemia versus 5-FO concentration at a given amount of PPMP.

PPMP treatment (M)	5-FO accumulation (10^{-3} nmol per 5×10^7 parasites)	IC_{50} (10^{-7} M)	
		[^{3}H]Hx incorp.	Giemsa stain
0	54	0.60	0.61
3.3×10^{-6}	4.5	7.5	7.4

export. PPMP does not inhibit amino acid uptake at the infected red cell membrane (*8*), suggesting that it blocks glutamate transport through the TVM. Importantly, the block in the transport of exogenous nucleosides and amino acids in PPMP-treated cells is not due to a reduced capacity for protein synthesis or export in these cells.

The transport of nucleosides by the TVM suggests that this network may be able to deliver toxic nucleotide compounds that have been of interest because of their potential antimalarial activities. Derivatives of orotic acid such as 5-fluoroorotate (5-FO) (*18*) are particularly promising because orotic acid is the only pyrimidine that is incorporated into the parasite. When PPMP-treated infected red cells were incubated with [^{14}C]5-FO (*19*), the accumulation of cell-associated radiolabel was inhibited by ~90% (Table 2), suggesting that the TVM is required for the parasite-induced transport of 5-FO. Furthermore, the TVM-arrested cells displayed more than a 15-fold increase in resistance to killing by 5-FO. These results raise the possibility that the TVM network may be exploited to facilitate the delivery of specific drugs (nutrient analogs) to the parasite. It is noteworthy that PPMP treatment does not affect the median inhibitory concentration (IC_{50}) of the antibiotic doxycycline (*19*), suggesting that the transport of an antimalarial drug is not invariably dependent on the TVM.

We provide evidence that the TVM appears to be a rate-limiting step in the transport of important nutrients such as adenosine, glutamate, and orotic acid to the parasite. A nonsaturable channel proposed to internalize a wide range of substrates (*20*) may function at TVM–red cell junctions. The TVM is distinct from the "duct" (*21*), a structure whose existence is debated (*4*, *5*, *22*) because it does not enable macromolecules to diffuse into infected red cells and it is not continuous with the red cell membrane (*6*, *10*). The TVM may provide a tubular endosomal network, and the channels that enable solute entry at TVM–red cell junctions could function as sieves. In TVM-arrested cells, the structures in association with the red cell membrane may import extracellular solutes. But subsequent solute transport to the parasite may be prevented as a result of fragmentation or constrictions in the network (or both). This may explain why arresting the TVM blocks the accumulation of exogenous substrates such as adenosine, orotic acid, and glutamate by ~60 to 80%, but prevents their incorporation into nucleic acids or proteins by ~90%. As a nutrient transport pathway, it is likely that the TVM is important only at the later stages of intraerythrocytic growth, and that alternate pathways dominate in the first 30 to 33 hours of the asexual life cycle. Additional parasite-induced nutrient acquisition pathways are the digestion of hemoglobin and a nutrient channel proposed to be in the parasitophorous vacuolar membrane (*23*).

Our results do not exclude the possibility that the TVM delivers antigens to the red cell membrane. The vesicular budding in the network may be sufficient to export parasite proteins to the red cell, whereas tubules provide the proper membrane conformation required to catalyze solute transport to the parasite.

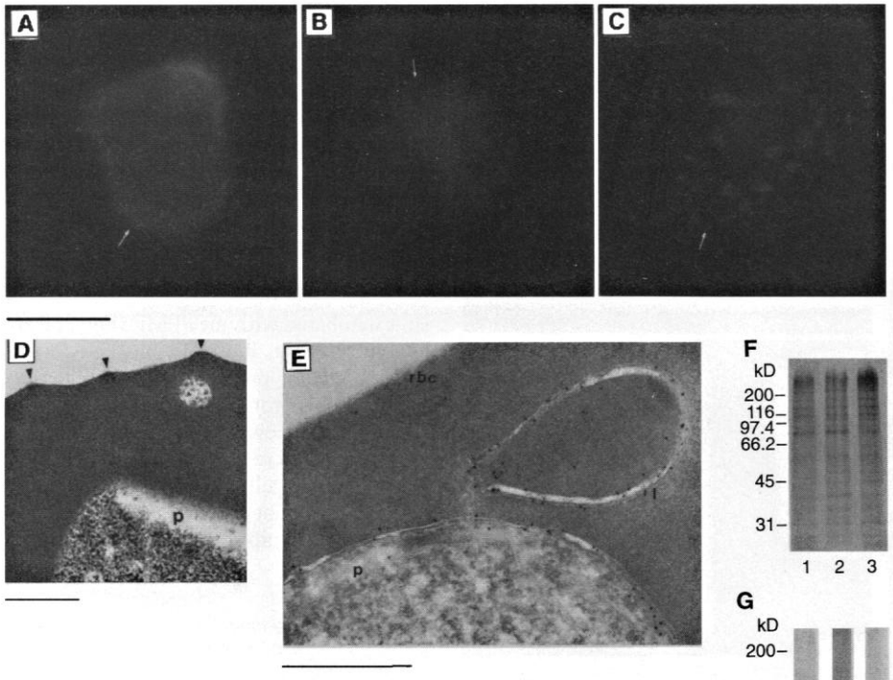

Fig. 2. Export of proteins by PPMP-treated parasites. Infected red cells treated with PPMP (for 36 hours, through ring and trophozoite development) were probed with antibodies to (**A**) PfEMP2, (**B**) PfHRP2, or (**C**) 45-kD Maurer's cleft protein and fluorescein isothiocyanate (FITC)–conjugated secondary antibody, in standard indirect immunofluorescence assays (*8*); (**D**) fixed, embedded in Polybed, and examined by thin-section electron microscopy (Phillips EM 201) (*8*) for knobs (indicated by arrowheads); and (**E**) prepared into ultrathin cryosections and probed with antibodies to Exp1 or preimmune antibodies (not shown) and goat antibody to mouse conjugated to 5-nm gold particles. In (E) the gold label is seen at the parasitophorous vacuolar membrane and the intraerythrocytic loop (l). p, parasite; rbc, red blood cell. Scale bar for (A) to (C), 5 μm; scale bar for (D) and (E), 0.5 μm. (**F** and **G**) Biosynthetic transport in PPMP-treated parasites released from the TVM and red cell membranes. Parasites were released and incubated (at 5×10^7 parasites/ml) with [^{35}S]Tran label (50 μCi/ml) for 60 min (*17*). Newly synthesized proteins associated with (F) or exported from (G) the parasite into the buffer were separated as described (*17*) and analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. Lane 1, control parasites in buffer alone; lane 2, parasites treated with 5 μM PPMP; lane 3, control parasites treated with 5 μM brefeldin A. The standard error between samples is ±10%. Molecular size standards are indicated on the left.

REFERENCES AND NOTES

1. G. Gronowicz, H. Swift, T. L. Steck, *J. Cell Sci.* **171**, 163 (1984).
2. J. A. Chasis, M. Prenant, A. Leung, N. Mohandas, *Blood* **74**, 1112 (1989).
3. K. W. Deitsch and T. E. Wellems, *Mol. Biochem. Parasitol.* **76**, 1 (1996).
4. A. M. Gero and K. Kirk, *Parasitol. Today* **10**, 395 (1994).
5. K. Haldar, *ibid.*, p. 393.
6. H. G. Elmendorf and K. Haldar, *J. Cell Biol.* **124**, 449 (1994).
7. B. C. Elford and D. J. P. Ferguson, *Parasitol. Today* **9**, 80 (1993).
8. S. Lauer, N. Ghori, K. Haldar, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9181 (1995). PPMP-treated cells were prepared by incubating purified schizonts (36 to 48 hours old) with a 20-fold excess of uninfected red cells for 12 to 16 hours in RPMI 1640 medium containing 10% human serum and 5 μM PPMP. This treatment produced a new generation of PPMP-treated infected red cells that were arrested in the TVM from the onset of ring development. Any residual schizonts were removed by a second Percoll gradient.
9. K. Haldar, A. F. de Amorin, G. A. M. Cross, *J. Cell Biol.* **108**, 2183 (1989).
10. K. Haldar and L. Uyetake, *Mol. Biochem. Parasitol.* **50**, 161 (1992).
11. B. Pouvelle, J. A. Gormley, T. F. Taraschi, *ibid.* **66**, 83 (1994).
12. To examine the uptake of LY after removal of PPMP, we washed cells in culture medium, allowed them to mature under normal growth conditions for 24 hours,

and subsequently incubated them with LY (9). To examine the effect of PPMP on the LY channel or transporter activity in the infected red cell membrane, we incubated trophozoite- and schizont-infected red cells with LY (9) in the presence or absence of 5 μM PPMP. The dye could be detected in both treated and control cells, indicating that PPMP does not block the channel or transporter activity at the infected red cell membrane.

13. A. M. Gero, E. M. A. Bugledich, A. R. P. Paterson, G. P. Jamieson, *Mol. Biochem. Parasitol.* **27**, 159 (1988).
14. PPMP-treated and control cells were adjusted to 2×10^7 cells/ml and incubated with a 1 μCi/ml concentration of [^{3}H]adenosine (34.5 Ci/mmol at a final concentration 29 nM) or [^{3}H]thymidine (2 Ci/mmol at a final concentration of 500 nM) in phosphate-buffered saline (PBS). Because of its low specific activity, the extracellular concentration of [^{3}H]thymidine was 16 times as high as that of adenosine. For the accumulation of orotic acid, infected erythrocytes treated with 5 μM PPMP (10 to 20% parasitemia) were washed free of serum and adjusted to 5×10^9 cells/ml in RPMI 1640. Transport was initiated by mixing equal volumes (330 μl) of the cell suspension and [^{3}H]orotic acid at a concentration of 0.26 μCi/ml (13 Ci/mmol at a final concentration of 10 nM). For the accumulation of glutamate, control and PPMP-treated infected red cells were adjusted to 2.5×10^8 cells/ml and incubated with [^{3}H]glutamate at a concentration of 1 μCi/ml (53 Ci/mmol at a final concentration of 19 nM) in PBS. For all accumulation assays, the cells were collected by centrifugation through a layer of dibutylphthalate, and the cell pellets lysed, bleached, neutralized, and counted. PPMP treatment had no effect on the uptake of any radiolabeled compound into uninfected red cells. The incorporation of adenosine and orotic acid into nucleic acids was measured as follows. Infected red cells treated with PPMP (5 μM) for 12 hours and their corresponding controls were seeded into microtiter plates at 2% hematocrit and 5% parasitemia. The cells were incubated in RPMI 1640 containing 10% human serum and 0.86 μCi/well (11 nM) of [^{3}H]adenosine or 0.125 μCi/well (48 nM) of [^{3}H]orotic acid in the presence or absence of (5 μM) PPMP for 24 hours at 37°C. All incorporation assays were carried out in the absence of NBMPR. For glutamate, infected red cells treated for 12 hours with PPMP (5 μM) and their corresponding controls were incubated for 24 hours at 37°C in RPMI 1640 lacking glutamate and supplemented with 2% human serum and [^{3}H]glutamic acid (20 μCi/ml, 377 nM).
15. J. L. Crary and K. Haldar, *Mol. Biochem. Parasitol.* **53**, 185 (1992).
16. B. C. Elford, G. M. Cowman, D. J. P. Ferguson, *Biochem. J.* **308**, 361 (1995).
17. H. G. Elmendorf, J. D. Bangs, K. Haldar, *Mol. Biochem. Parasitol.* **52**, 215 (1992).
18. P. K. Rathod, A. Khatri, T. Hubbert, W. K. Milhous, *Antimicrob. Agents Chemother.* **33**, 1090 (1989).
19. Determination of the IC_{50} of 5-FO in TVM-arrested cells by hypoxanthine incorporation was carried out by plating infected red cells in microtiter dishes at 1% hematocrit and 1% parasitemia. [^{3}H]Hypoxanthine (0.5 μCi/well) was added, and the cells were incubated for another 24 hours and then harvested on glass fiber filters (8). Because PPMP reduces the accumulation of exogenous [^{3}H]hypoxanthine (but does not inhibit the parasite's machinery for nucleic acid synthesis), a separate IC_{50} plot for 5-FO was determined at 0, 0.03, 0.3, and 3.3 μM PPMP. At 0 to 0.3 μM PPMP, the IC_{50} was 6.0×10^{-8} M. At 3.3 μM PPMP (which corresponds to complete inhibition of the SSS and the TVM), the IC_{50} of 5-FO was 7.5×10^{-7} M. As expected, saponin abrogated the effects on nucleoside uptake. Determination of the IC_{50} in TVM-arrested cells by Giemsa staining was carried out with infected red cells at 2% parasitemia and 5% hematocrit. Cells were subsequently washed free of both PPMP and 5-FO and the parasites were allowed to grow in RPMI 1640 for another 48 hours, at which time parasitemia was determined by Giemsa staining. The IC_{50} of doxycline (in the absence or presence of PPMP) was 1×10^{-6} M. These experiments were carried out as those described for 5-FO.
20. K. Kirk, H. A. Horner, B. C. Elford, J. C. Ellory, C. Newbold, *J. Biol. Chem.* **269**, 3339 (1994).
21. B. Pouvelle *et al.*, *Nature* **353**, 73 (1991).
22. T. F. Taraschi and E. Nicholas, *Parasitol. Today* **10**, 399 (1994).
23. S. A. Desai, D. J. Krogstad, E. W. McClesky, *Nature* **362**, 643 (1993).
24. J. R. Swedlow, J. W. Sedat, D. A. Agard, *Cell* **9**, 97 (1993).
25. We thank W. I. Li, J. McBride, D. Taylor, and R. Coppel for antibodies to the 45-kD cleft protein, Exp1, HRP1, HRP2, and PfEMP2; A. A. Holder for a knob-forming FCB strain of *P. falciparum*; R. R. Kopito and S. Mayor for comments on the manuscript; and S. Palmieri and J. VanWye for assistance with the Delta Vision microscope and work station. Supported by NIH grants (AI26670 and AI39071 to K.H., AI26912 and AI01112 to P.K.R.), the MacArthur Foundation (S.L.), and Burroughs Wellcome Fund Awards (New Investigator and New Initiatives in Malaria to K.H.).

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Engineering Chemical Reactivity on Cell Surfaces Through Oligosaccharide Biosynthesis

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Cell surface oligosaccharides can be engineered to display unusual functional groups for the selective chemical remodeling of cell surfaces. An unnatural derivative of *N*-acetylmannosamine, which has a ketone group, was converted to the corresponding sialic acid and incorporated into cell surface oligosaccharides metabolically, resulting in the cell surface display of ketone groups. The ketone group on the cell surface can then be covalently ligated under physiological conditions with molecules carrying a complementary reactive functional group such as the hydrazide. Cell surface reactions of this kind should prove useful in the introduction of new recognition epitopes, such as peptides, oligosaccharides, or small organic molecules, onto cell surfaces and in the subsequent modulation of cell-cell or cell–small molecule binding events. The versatility of this technology was demonstrated by an example of selective drug delivery. Cells were decorated with biotin through selective conjugation to ketone groups, and selectively killed in the presence of a ricin A chain–avidin conjugate.

Cell surface molecules govern many biological events such as cell growth and differentiation, communication among different cells, recognition of soluble factors, and attachment to, or disengagement from, the extracellular matrix. Technology that orchestrates the presentation of chemically defined epitopes on a cell surface would enable direct intervention in these biological processes. We present a strategy for remodeling the cell surface in a chemically defined way by exploiting the substrate promiscuity of oligosaccharide biosynthetic pathways. The natural metabolic processes of the cell were used to introduce a reactive functional group, the ketone, into cell surface–associated sialic acid residues. The ketone group is virtually absent from cell surfaces in that none of the naturally occurring amino acids, glycoconjugates, or lipids contains a ketone group, and it can be chemoselectively ligated with hydrazide, hydroxylamino, and thiosemicarbazide groups under physiological conditions (*1*–*5*). Thus, the ketone provides a molecular handle for the attachment of biomolecules or small molecular probes to cells. Because the display of ketone groups is achieved solely through the action of the biosynthetic machinery, this process constitutes a practical method to engineer the composition of the cell surface in vivo.

Sialic acids are appropriate vehicles for cell surface functional group display because (i) they are the most abundant terminal components of oligosaccharides on mammalian glycoproteins and glycolipids, and (ii) the enzymes that participate in sialic acid metabolism are permissive for simple unnatural substrates (*6*–*10*). Sialic acids are biosynthesized from the six-carbon precursor *N*-acetylmannosamine (ManNAc) (*11*, *12*). Reutter and coworkers have demonstrated that unnatural mannosamine derivatives, in which the *N*-acetyl group of ManNAc was substituted with *N*-propanoyl, *N*-butanoyl, or *N*-pentanoyl, are converted to the corresponding sialosides and incorporated into glycoconjugates in cell culture and in rats (*13*, *14*). These observations suggest that all components of the cellular machinery involved in sialoside biosynthesis can tolerate unnatural acyl substituents.

Accordingly, we synthesized *N*-levulinoyl mannosamine (ManLev) (Fig. 1A), which has the ketone functionality at the position normally occupied by the *N*-acetyl group in the natural substrate

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