Homework due at the start of class on July 3

Read "Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance" (D.A Fidock et al., *Molecular Cell* **6**: 861-71, 2000; PubMed ID 11090624) and do the assignment below, consulting any additional sources as needed. You may be able to access the full text of the article by going to http://pubmed.gov, doing a search for the PubMed ID, and following a link from the article's abstract page to the publisher's website. Alternatively, you can access the article online from the E-Journals section of the UW Libraries website, as described in previous homework assignments.

General background

With this article we conclude a two-part series addressing the question of "Why do we need new malaria drugs?" The simplistic answer is (1) malaria vaccines are not very effective (the topic of the previous paper) and (2) the *Plasmodium* parasite is good at evolving resistance to current drugs (the topic of the current paper).

As you'll see, the research described by Fidock et al. was done before a complete draft of the *Plasmodium falciparum* genome was available. (The preliminary analysis of this genome was published in October of 2002.)

A bit of relevant cell biology: the **parasitophorous vacuole** is the compartment within infected mammalian cells (hepatocytes or erythrocytes) where the *Plasmodium* parasite resides. Within parasite cells, the **digestive vacuole** (DV; also called a food vacuole) is a compartment where hemoglobin from the host is digested. Do not confuse these two vacuoles, as I once did! The Wikipedia entry "*Plasmodium falciparum* biology" has more information on both.

You all are familiar with notation of the form "K76I," right? This means that the 76th amino acid is normally K (Lysine) but has been mutated to I (Isoleucine).

Don't be overly distracted by the fact that chloroquine resistance (CQR) is "verapamil-reversible." This point is interesting, but not especially important for our purposes. One 2010 paper (PubMed ID 20807203) offers the following explanation: "Two theories have been proposed for VP reversal of CQR: (i) the 'drug transporter' hypothesis argues that VP competes with CQ for target binding and perhaps drug transport in resistant parasites (Martiney et al., 1995; Bray and Ward, 1998; Sanchez et al., 2004), and (ii) VP may act on ion transport to alter the compartmental pH and/or membrane potentials to levels found in CQS parasites (Martiney et al., 1995; Roepe and Martiney, 1999; Ursos et al., 2000; Bennett et al., 2004). Both hypotheses are possibly relevant, as drug transport by drug resistance proteins could be due to direct and/or indirect ion-mediated co-transport (Zhang et al., 2004)."

Note that genetic crosses were made as part of this study. Recall the information on the *Plasmodium* life cycle that you looked up as part of the previous homework assignment; in particular, note that the parasite is a haploid organism throughout the human stages of the life cycle. While the methods for doing genetic crosses with this parasite are not clearly laid out in the present article, an earlier article (PubMed ID 3299700) says, "The parasites can be analyzed

genetically by transmitting mixtures of cloned parasites through mosquitoes to permit cross-fertilization of gametes to occur…. Parasites showing recombination between the parent clone markers were detected at a high frequency."

Fidock et al.'s general strategy is typical microbiology in some ways. To attribute a given phenotype to a given gene, one often begins by observing where mutations have spontaneously occurred, but the real proof often comes in introducing supposedly critical mutations into a wild-type organism and showing that those are necessary and sufficient for the phenotype to change. For genetic changes introduced via plasmids, typical experiments include transfecting cells with a plasmid either containing the gene of interest or not containing it, and letting the plasmid disappear from the cells (e.g., by removing the antibiotic that was previously forcing the cells to keep the plasmid), and seeing whether the phenotype tracks with the gene in each case.

Worksheet to hand in

1. name, date, and assigned article

Greg Crowther, 7-7-13, "Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance"

2. In brief, how does chloroquine kill *Plasmodium* parasites? Consult any reliable online source or textbook. (2-3 sentences)

Parasites break down the oxygen-binding protein hemoglobin and use its amino acids for building new proteins. The breakdown of hemoglobin causes the release of the prosthetic group heme, which is toxic to the parasite. (The exact reasons for this are not obvious to me, though one paper [PubMed ID 21458590] refers to its "redox and detergent-like properties.") Parasites normally aggregate heme into insoluble crystals, thus neutralizing its effects, but chloroquine interferes with this process, causing a buildup of the toxic heme groups.

3. Briefly explain how PCR of cDNA libraries can be used to find previously unidentified transcripts, as mentioned in the first paragraph of the results. (2-3 sentences)

A cDNA library consists of mature mRNA transcripts converted back to DNA by reverse transcriptase. When you harvest the mRNA from cells, you don't know what sequences are included and not included in the mRNA, but you can check whether any particular stretch of DNA is included by doing PCR on the cDNA with appropriate primers and then sequencing the amplified region.

4. Figure 1 shows 10 predicted transmembrane regions. This prediction of 10 transmembrane segments is attributed vaguely to "database searches, alignments, and prediction algorithms." Find out a bit more about how transmembrane domains can be predicted from amino acid sequences. How do they work? (~2 sentences)

The most basic algorithms are based simply on running averages of hydrophobicity; hydrophobic stretches of amino acids (often about 20 amino acids long) were predicted to be

transmembrane domains (TMDs). Also, now that many transmembrane proteins have been characterized, TMDs of less-studied proteins can be predicted according to homology to the TMDs of the well-studied proteins (i.e., the amino acid sequences are similar).

5. Fidock et al. report (in the bottom of the left column of p. 862), "The remaining clone, 106/1, a previous exception to complete association between cg2 and CWR (Su et al., 1997), was particularly informative." Why did they consider this clone "particularly informative"? (1-2 sentences)

This clone highlights the importance of the K76 amino acid. 106/1 has other mutations matching those of chloroquine-resistant African and SE Asian strains, but has K76 and is chloroquine-sensitive, meaning that mutation of K76 to something else is necessary for chloroquine resistance.

6. Table 1 lists many chloroquine-sensitive and chloroquine-resistant cell lines. Do we know whether the lines clearly cluster into "sensitive" and "resistant" responses to chloroquine, or is there more of a continuum? Is it important for us to know this? (2-3 sentences)

This paper doesn't show whether the distinction between CQR and CQS strains is clear-cut. However, Su et al. 1997 (cited by this paper) does have a table showing IC50s (concentrations needed for 50% inhibition of growth to occur), and those data show CQS groups having IC50s of 2-9 ng/mL and CQR groups having IC50s of 40-120 ng/mL. This may help us understand that each of the mutations shown in Table 1 are apparently not contributing an incremental increase in chloroquin resistance; rather, all of the mutations must be present simultaneously for true chloroquin resistance to occur.

7. Table 2 concerns measurements of *P. falciparum* growth via microscopy on blood smears (counting the percentage of erythrocytes that are infected, i.e., percent parasitemia) and via measurements of the uptake of radioactive hypoxanthine (a purine that gets incorporated into the DNA of growing/dividing cells). What is/are the main 1-2 points of Table 2? (Hint: look at the sentences in the text that refer to Table 2.) Which particular values in Table 2 make these points? (2-3 sentences)

(I'm sorry that hint wasn't very helpful; Table 2 is cited in 10 different places! In this case, looking at subheadings may be your best way of quickly figuring out what is going on.)

As mentioned in the "General background" section of this study guide, to prove that the specific mutations in the pfcrt gene are responsible for chloroquine resistance, you can introduce the mutated gene into chloroquine-sensitive cells and see whether they become chloroquine-resistant. This is the essence of Table 2. Adding plasmid pNHSC (including a pfcrt for chloroquine resistance) to 106/1 cells increased the average IC_{90} values from 42 nM to 78-201 nM; adding plasmid pDC/CRT-Dd2/trunc. to GC-03 cells increased the average IC_{50} from 31 to 53 nM and the average IC_{90} from 40 to 87 nM.