Homework due on July 14 (yes, *Sunday*!)

Read "High-throughput *Plasmodium falciparum* growth assay for malaria drug discovery." (M.L. Baniecki et al., *Antimicrobial Agents and Chemotherapy* **51**: 716-23, 2007; 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.

Use the study guide below to help you understand the article. Then by *Sunday, July 14 at 8:30pm*, please email me (crowther@u.washington.edu) one question about this article that you think would be a good basis for an in-class discussion. Your question should be about the section of the article assigned to you according to the table below. Your question should be hard enough to be worthy of discussion, but still potentially answerable by someone seeing the question for the first time.

Arthur, Jennifer Lynn (Jen): results	Hammond, Lesley J: results
Asif, Romaisa: results	Isakharov, Emoniel Mikhail: results
Barber, Jennifer N: methods	Lam, Michael: methods
Boevers, Emily A: methods	Lanphere, Margaret Mimieux: results
Bradshaw, Tyler Wesley: introduction	Ngo, Tracy My: methods
Choe, Dongwook Christian (Chris): methods	Nguyen, Richard Duc Phuoc: introduction
Clough, Courtnee Amanda: discussion	Novak, Avrey Ann: methods
Cristel, Ripp Royale: introduction	Olson, Julia Renee: results
Dela Cruz, Kristi Jean Camacho: methods	Saiku, Daniel Akira: discussion
Foucher, Clementine Marguerite: methods	Stockdale, Graham G: discussion
Franz, Christopher Douglas (Chris): introduction	Sumulong, Chad Racpan: results
Giddings, Kimberly Ashlyn Avery (Ashlyn): results	Valshon, Kaiser David: discussion

General background

Note that this study is based on phenotypic or cell-based screening (i.e., find compounds that kill the pathogen, and figure out how they work later), as opposed to target-based screening (find compounds that affect the function of a particular target, and later determine whether modulation of that target can kill the pathogen).

Abstract

The final sentence notes, "181 small molecules were identified as highly active against multidrug-resistant parasites." That is, the researchers performed their screens with *P. falciparum* strains HB3 and Dd2, which are resistant to various drugs (see Table 2 later in the article and the bottom right of p. 721), as well as the standard drug-sensitive lab strain 3D7. In searching for antimalaria compounds, you want to find compounds that are good at killing drug-resistant strains that are representative of what's out there in the real world.

Introduction

The paragraph beginning at the end of page 716 lists the (probable) targets of current antimalaria drugs. The very limited number of them could be interpreted in an optimistic way ("Wow, there are lots of good targets that haven't been exploited yet – let's study them!") or more pessimistically ("Geez, the number of targets that are actually exploitable must be much lower than we think"). Also note that the drugs listed were discovered and developed without knowledge of their specific targets, which came later. Thus the target-based approach has yet to prove valuable in the malaria community. For more on this topic, see the last full paragraph of page 722.

As you know, *Plasmodium* parasites invade erythrocytes (red blood cells) and therefore are commonly grown in the lab using red blood cells. DNA staining is a useful way of quantifying parasite growth (see the last two paragraphs of the Introduction) because red blood cells have no DNA.

Note that Baniecki et al. cite prior studies of staining of parasite DNA, including at least one (#17) that claims to be HTS-compatible, but then add, "These methods are not sufficiently robust." This diss is partly explained in the first paragraph of the Results (p. 718) and the third paragraph of the Discussion (p. 722). Note that heme is present in high concentrations in (both infected and uninfected) red blood cells, so a high background signal from heme is indeed a problem.

Materials and Methods

Try not to get too bogged down in the methods section. For both this section and the Results, use the bold-faced subheadings to see how the information is organized, but don't obsess over all of the details.

"Cultivation of *P. falciparum*": It's good to know what ingredients are necessary to grow *Plasmodium* in culture, so read this over. Albumax contains synthetic bovine serum albumin similar to that found in blood. Gentamycin is an antibiotic for preventing contamination of the cultures. Hypoxanthine is a purine used by the parasite to make purine nucleotides for nucleic acid synthesis, a process that is the basis of the [^{3}H]hypoxanthine method discussed below.

"DAPI *P. falciparum* growth assay HTS design": Don't worry about all of these details. Figure 1 is a good summary. Also, make sure the formula for calculating the percentage of parasite growth in the test wells (about two thirds of the way down the right-hand column) makes sense to you. $CQ(LD_{90})$ represents the minimum signal – the signal that you'd get from cells that aren't dividing at all (because they're exposed to high chloroquine concentrations) – whereas Avg_+ is the maximum signal – the signal from parasites growing in the absence of any drug.

"Assessment of the percentage of *P. falciparum*-infected erythrocytes": Note that three different methods of quantifying growth were used. The method of [^{3}H]hypoxanthine incorporation into DNA is a well-known and trusted technique against which the newer fluorescence-based methods were compared. The reasons for looking at both "fluorimetry-based" and "imaging based" approaches are mentioned in the fourth paragraph of the Discussion.

Results

As in the Methods section, a lot of information is presented here. Try to keep the "big picture" in mind, using the bolded subheadings as a summary of the overall progression of the study.

To validate the new DAPI-based methods of quantifying parasite growth, the researchers needed to show that they give results comparable to those obtained with an already-trusted method ([^{3}H]hypoxanthine

incorporation). This validation took place in two steps. First, in the absence of any drugs, the fluorescence measurements were shown to correlate closely to the [^{3}H]hypoxanthine data (Fig. 2). Second, all methods were shown to yield similar IC_{50} values for known antimalarials (Table 1).

Page 721 says that the compounds were screened at concentrations of "approximately" 30 µM and 6 µM. Why do you think these concentrations were considered approximate? A couple of possibilities are that (a) they didn't have enough of some compounds to make stocks yielding final concentrations of 30 and 6 µM, so lower concentrations were used, and (b) some compounds weren't very soluble, so the concentrations of these compounds in solution may have been lower than 30 and 6 µM.

Discussion

The third paragraph makes an interesting point about the emission and excitation wavelengths of heme. However, note that many compounds in chemical libraries have absorption and emission wavelengths similar to those (355/460) used in the DAPI assay. This limits the DAPI assay's ability to measure growth inhibition by compounds that fluoresce in this region of the spectrum.

The final paragraph lists the "five hallmarks of a successful high-throughput screen": robustness, reproducibility, technical simplicity, suitability for automation…. Hmm….

Students' questions submitted for in-class discussion of Baniecki et al. (2007)

The task of submitting questions for in-class discussion was intended partly as a warm-up for leading full-blown discussions in the coming weeks. In some of my comments below, I say that a question might not have fit well into my usual format for small-group work, or that I wasn't sure what answers you were looking for. However, a question that didn't quite work for me might work great for you! When you're in charge of a discussion, you can structure that discussion as you wish (including adjusting the time given to small groups, if small groups are used), and you can clarify ambiguities on the fly, if needed. Perhaps most importantly, when you're in charge, you get to decide on which content to prioritize, whereas in this case I used questions that fit my own priorities.

All of that aside, I really enjoyed reading your questions and gave full credit to everyone who submitted one on time.

Create a flow diagram in order to explain how a high-throughput screening assay could be used to discover new antimalarial drugs. Consult Fig. 1 if necessary.
Greg's comments: Good question, except that I wasn't sure whether the flow diagram was supposed to go beyond this experiment, showing further progression to animal/human studies. Was the idea to have people create an alternative version of Fig. 1, or to put Fig. 1 in a broader context?
What does the author mean when he says that the new DNA staining methods are "not sufficiently robust" for use in HTS?
Greg's comments: I liked this question. My only concern was that discussing it might not fill the usual time I allocate to small-group work (10-12 minutes). If a follow-up question had been added, it might have fit the time slot quite nicely.
In the first paper we read, J. Drew writes that every disease has between 5 and 10 genes that contribute to its trait and each gene has somewhere between 5 and 10 proteins that are targetable. By simple multiplication, let's assume the range of *potential* drug targets for a particular disease is somewhere between 25 and 100 and that this holds true for malaria. Use the fact that there are finite "targets" (as described above), and information in the introduction given in the paper "High-Throughput Plasmodium falciparum Growth Assay for Malaria Drug Discovery" by M. L. Baniecki, et al. 2007 and what you already know about malaria and to formulate an argument for allocating resources towards finding a vaccination for the malaria as apposed to new drugs designed to treat malaria.
Greg's comments: This is a very interesting question. My main concern about it was that it seemed fairly sprawling and all-encompassing, so the usual amount of time that I've been devoting to small-group work (10-12 minutes) didn't seem adequate for this. It would be interesting to work through as a whole class and/or over a somewhat longer period of time.
In the introduction, they list the four classes of antimalarial compounds: quinine or aminoquinolines, antifolate compounds, arte- misinin derivatives, and hydroxynapthoquinone atovaquone. They say, "This lack of structural diversity means that previously developed therapeutic alternatives, really modifications of the same basic molecular templates, might prime new drug candidates for the rapid emergence of resistance.". What do they mean by "lack of structural diversity" in these chemical compounds?
Greg's comments: Good question! I used it in class. About the right level of difficulty – not too

obvious, but not impossible.

What does the LD90 value represent? How is this value used in determining a "hit" or a screening positive?

Greg's comments: Good question! Although I didn't use it in class, I certainly could have done so. It's about how you decide which compounds are your hits, which is a central issue of any screen.

What is the significance of studying the 'ring stage' of the P. falciparum erythrocyte infection? Would there be a benefit to looking at another type of infected cell or another stage of infection?

Greg's comments: Good question. This issue was not one I particularly wanted to emphasize in this paper, but it would have been interesting to talk about.

Within the context of the paper, which removal from the methods section would have changed the discussion section the least? Removal of the positive control, or removal of the negative control?

Greg's comments: I found this question kind of tricky, but very interesting! It's great to imagine how the experiments could have been set up differently, and what the consequences would be, and I like how this question forces us to do that.

What were the three different methods used for quantifying parasite growth and how did they differ in terms of method and what was measured? What were the advantages of using the less traditional, trusted and more recent methods?

Greg's comments: Very good question. I used it in class. A key part of the paper is the comparison of the "traditional" hypoxanthine assay with the newer fluorescence-based assays, and this question focuses appropriately on that.

What is the purpose of synchronizing P. falciparum cultures, and why do the researchers prefer the ring-form stage for drug susceptibility tests?

Greg's comments: I didn't use this partly because I personally didn't want to emphasize these issues in this paper, and also because I thought further research beyond this paper (sometimes hard to do on the fly in class) might be needed to answer the question. But I still think it's a good, interesting question.

Having read the article in terms of how the scientists went in cultivating their Malaria assay what are some of the concerns that may arise from sending samples to multiple places for data?

OR

Was every step of the set up justified within the article? In terms of level to set the centrifuge and other factors what could have been done to better make the article more user friendly?

Greg's comments: The first question seemed interesting, but I wasn't sure what you were looking for in terms of an answer. I love the idea of the second question – going over the methods and figuring out what could have been changed to make the article more user-friendly. I wasn't sure whether 10-12 minutes would be sufficient for that task, though.

- What is the difference between the fluorometric and imaging detection techniques? Why do we do both?

- Why did the researchers choose use the 384-well microtiter plates?

Greg's comments: The first question is good, covering a central issue of the paper (comparison of the two techniques). I used a similar question (submitted by someone else) in class. I wasn't quite sure of the intention of the second question (i.e., whether the answer was "to screen lots of compounds quickly," and/or something else).

What role did hypoxanthine play in the cultivation of *P. falciparum*? Explain how parasite growth was measured using [^{3}H]hypoxanthine incorporation.

Greg's comments: This was an important methodological issue that is definitely worth

covering. The version of this I used in class was just a bit broader, asking people to compare the hypoxanthine method to the fluorescence-based methods.

Figure 4 shows screening positives for compounds inhibiting three different P. falciparum strains. A compound was considered a positive if it inhibited one strain of parasite growth by greater than 90% (LD 90). Why do you think the compound was considered to to be a positive for two or more strains if the first strain showed a LD 90, but only a LD 10 for the other strain(s)?

Greg's comments: The mention of LD10 in the Fig. 4 legend is very confusing. I think this question may reflect some confusion about that. The general point of the Venn diagram is just that a few compounds did not inhibit growth of all 3 strains, and so those are considered "selective" for 1 or 2 strains.

What was the purpose behind a secondary screening of 900 screening positives identifies in primary HTS? How was this achieved? (Why was concentration level manipulated?)

Greg's comments: Good question covering a central issue in screening, i.e., how do you follow up on the compounds that initially come up as hits in your screen. I couldn't use this question in class because it was submitted late.

What evidence was presented that the DAPI staining technique was effective in HTS? How would you present this data if you were writing the paper?

Greg's comments: Good question. I used it in class because I like the way it asks us to consider alternative ways of presenting the data.

Wouldn't immunofluorescence microscopy only be indicative for enhancer elements, and exclude any potential silencing elements in transcriptional control of genes?

Greg's comments: I was confused by this question. This study did not involve immunofluorescence as far as I can tell, in that no antibodies were used. Fluorescence was indeed detected by microscopy, but this fluorescence should have simply reflected staining of DNA rather than control of transcription.

Given the information presented to us in the results section, do you feel that you were convinced that the DAPI *P. falciparum* growth assay HTS design is just as good, if not better, version of target detection as the ([3H]hypoxanthine) method?

Greg's comments: A good question, covering the key issue of how the hypoxanthine and fluorescence-based methods compared to each other. I couldn't use the question in class because it was submitted late.

What was the point of using different parasite phenotypes (3D7, HB3, DD2) to gain results? Would it have been sufficient to use a highly resistant strain (I think DD2 was found to be resistant to chloroquine, melloquine, and pyromethamine) to get the best results for the new hits and more possible drugs? Or was this research solely just to experiment with using DAPI for HTS assays?

Greg's comments: I liked this question and used it in class. I can imagine more than one reasonable answer, which is always good for stimulating good discussion.

The paper mentions that "whole-organism assays offer possibilities not available to target-based assays." What are the pros and cons that the authors list with such assays and do you agree with their list?

Greg's comments: I used this question in class because I liked it. Having previously talked about target-based and whole-organism assays, I felt that we had enough background to evaluate the authors' list, as suggested.

Target based approaches to identifying new anti-malarial drugs have resulted in zero discoveries. Is this approach useless to the malaria community or are there some benefits to research that this approach provides?

Greg's comments: A great question worthy of discussion. I didn't use it because it is not about this paper, per se, as much as some other questions were.

So this DAPI stain-based P. falciparum whole-organism growth assay measures the level of blood-stage parasites. However, from my understanding of the "life-cycle" of the parasite, the first state of infection is in the liver/hepatocytes. While the article claims that this "whole growth" assay is highly robust it mentions nothing about identifying the parasite at the liver-stage. Is it premature to call an assay robust if it only identifies one stage of the infection cycle?

Greg's comments: Very interesting question, but one that might have sent us away from my highest-priority concepts, i.e., I wasn't planning to discuss other life-cycle stages in the context of this paper.

If cell-based screening offers results more relevant to real-world conditions, why bother doing target-based screens of large compound libraries?

Greg's comments: Good question. Some researchers actually feel that doing target-based screens is foolhardy, so I would have rephrased the question as, "Since cell-based screening seems to offer results more relevant to real-world conditions, should people bother doing target-based screens of large compound libraries? Why or why not?"