Slide 2

CMV Resistance – Typical Setting

As with our last talk, let us begin with a case where CMV drug resistance became a management problem. This case was published earlier this year because I got involved in studying a new mutation that showed up. However, the case actually illustrates a number of other features of interest and shows a somewhat typical setting, where the management of resistance becomes problematic.

We have a 50-some-year-old with AML in relapse who received an allogeneic bone marrow transplant who was treated subsequently with tacrolimus-prednisone and then following the preemptive treatment protocol, was started on valganciclovir after a first detected antigenemia, some 50 days after the transplant.

This seemed to go well initially. As you can see, the antigenemia went from 16 to 0, undetectable, and all seemed to be well for a while. Then the patient started to develop some abdominal symptoms – pain, diarrhea – which actually caused an endoscopy to be done. You could argue that during this period, because of the GI symptoms, the absorption of the valganciclovir could be in question. In fact, that raises the question of when you are giving drugs orally, you may have to be aware of whether they are being absorbed properly.

Even though there was no positive finding on the endoscopy, the antigenemia started rising again. Because of this concern, the patient was admitted and started on intravenous foscarnet.
CMV Resistance – Typical Setting

This was given for only a short period of time because of intolerances as described – probably electrolyte and renal function, as is typical for this drug. So the patient was then switched to cidofovir.

This was not a successful treatment because the antigenemia rose to a maximum of greater than 200, and this was not at all affected by the cidofovir. This is not the first case in which I have been involved where cidofovir appeared not to do the intended job in the setting where you have increasing viral loads and impending disease.

Because of this lack of apparent activity, the patient was switched back to higher-dose IV ganciclovir on the grounds that perhaps a higher level of the drug might be therapeutically effective. However, this, too, seemed to lack a therapeutic effect because the patient, while on this treatment, developed evidence of CMV pneumonia with hypoxemia and despite the administration of IVIG, and starting foscarnet therapy belatedly, the patient went on to die of respiratory failure and CMV pneumonia. You will notice here that you have this paradox, which has been noted before, where you see the antiviral effect of the foscarnet, ie, the antigenemia load has gone to 0 from 200, but it is too late to save the patient.

I do not know whether they received this genotype information in real time or not. A UL97 mutation was indeed seen in connection with this case, and this was a deletion of codon 601 to 603. This is in a region of UL97 that we have previously known to be the location of many ganciclovir-resistant mutations. However, not this specific one. If this had been known in real time I think people could have reasonably inferred that it was probably related to resistance, as was subsequently proven and was a subject of this publication.

With that, these couple of next slides can be brief because this is purely introductory material that has already been mentioned previously. The important things to note are that our current CMV antivirals are all viral DNA polymerase inhibitors; therefore, mutation in the viral DNA polymerase gene can confer resistance to one or all of these drugs. The special situation with
regard to ganciclovir and acyclovir is that these drugs require activation by phosphorylation at the position indicated, and that in the case of CMV, this is accomplished by the UL97 kinase.

Slide 3

Current CMV Antivirals

You notice here the structural difference between ganciclovir and acyclovir is the extra group that is shown in red. We are all aware of these adverse effects that accompany the use of these classic drugs that have been around for a long time.

Slide 4

Risk Factors for CMV Drug Resistance

Risk factors for CMV. Drug resistance. A reminder from our previous talk. We just went over these things. The listing of risk factors then includes prolonged drug exposure, usually months; immunodeficiency; and in the transplant situation, specifically the D+R− subset. Suboptimal antiviral drug activity, as may have played a role in the case just illustrated, such as missed doses, oral bioavailability, absorption, and such.

Increasing viral loads during treatment could be suggestive of drug resistance if the patient has been on the drug for a significant period of time. However, we clearly have encountered cases where rising CMV loads are not due to drug resistance. Therefore, in order to convincingly diagnose drug resistance, you do need to confirm it in the laboratory by some sort of testing.

Slide 5

CMV Resistance – Phenotypic Assays

The most traditional testing for CMV drug resistance are these phenotypic assays. They seem to be getting more and more old-fashioned as we talk about this subject. Some years ago it was the first thing you do. Today, there are practical difficulties in obtaining our viral isolates, and that has changed practice a little.

You have to get a viral isolate and cell culture and determine the IC50, which is the drug concentration that inhibits the virus by 50%. In order to make that determination, though, you
have to calibrate the assay, which is difficult and slow, because the virus grows slowly. Nowadays it is often not even available because viral isolation is not commonly performed. A calibrated inoculum is required. You cannot just estimate some dose going into the assay. The quantitation assays themselves are relatively inefficient, especially if you use the traditional plaque-counting method. The growth is affected by the cell culture condition. All of these things combine to give you this situation that the results from phenotypic testing are simply not fast enough to guide clinical decisions.

These assays do, however, reveal that most ganciclovir-resistant, or in general drug-resistant, CMV isolates have 2- to 10-fold increased IC50 over a baseline-sensitive virus. Now the level of resistance can be higher if multiple viral mutations accumulate over time. The resistance levels can go up to several 10-folds, which clearly would take it out of the therapeutic range of any of our current drugs.

**Slide 6**

**CMV Resistance – Genotypic Assays**

Nowadays more commonly we go with the genotypic assays because of the possibility of faster turnaround and of getting data in time to make decisions. So the way this is done is to amplify, by PCR, UL97 and DNA polymerase sequences from an isolate, or more commonly, direct from the clinical specimen. Then certain mutations are checked for and, of course, specifically those that have been proven to be linked to drug resistance.

**Slide 7**

**CMV UL97 Kinase Mutations**

In UL97 we have mutations popularly located at codons 460-520 and in the range of 590 to 607. These mutations affect ganciclovir only. UL97 mutations do not affect sensitivity to foscarnet or cidofovir.

The UL97 mutations are so focused that only the three or four most popular ones can account for two thirds of all of them. There have been attempts to focus the testing on the most common
CMV UL97 Kinase Mutations

mutations. But today, just to be complete, we usually do sequencing that covers these codons so that we can detect the entire range of mutations that have been validated in the UL97 gene.

In the DNA polymerase gene a wide range of codons can be affected when there is mutation to drug resistance. There can be varying patterns or combinations of resistance for one or more of the current drugs. These mutations will have to be checked against amino acid changes of known significance, that is, the database that exists today, which is constantly growing.

When mutations are detected, they are usually approximately at 20% or higher of the total viral population. Smaller percentages of mutant virus will probably escape detection.

The turnaround time of less than 1 week may improve clinical decision-making. Not all medical centers have access to testing, which will get you the results in less than 1 week. But, increasingly, commercial laboratories try to get these assays back to you somewhere between 1 and 2 weeks.

Now, in detail, a review of the two genes, UL97 and polymerase. UL97 actually has an important normal function in the life of the virus. It is a serine-threonine kinase that is essential for the normal release of infectious virus. It phosphorylates multiple targets of probably both viral and cellular origin. It is not yet known which substrates of UL97 are the most significant. However, if you do not have functional UL97, viral precursors will accumulate abnormally, especially in the nucleus of infected cells, and somehow hold up the proper assembly and release of infectious particles.

The incidental function of UL97, the nonbiological function, is to phosphorylate acyclovir and ganciclovir. Ironically, this was the first discovered function of UL97 and predated a lot of the recent work on UL97 knockouts and kinase inhibitors.
CMV UL97 Kinase Mutations

The location where ganciclovir binds UL97 is inferred from the mutations that we see in ganciclovir-resistant isolates. And as I mentioned before, codons 460-520 and 590-607. These map to locations in the gene, which are probably more involved with substrate recognition than in the core functionality of the kinase.

Every protein kinase has certain domains that are highly conserved, and this includes the glycine-rich domain 1, subdomain 1, which is involved in ATP binding, and the codon 460 is actually right in the middle of 6B, which is involved in the actual phosphoryl transfer that is part of the kinase activity.

When we interpret mutations in UL97, often I will be asked about certain other amino acid changes that happen to be found in clinical isolates. Some of the more popular ones are H469Y, B605E. These repeatedly come up for discussion because they are found in clinical isolates and the question is, are they related to drug resistance. These, for example, were discovered in a survey that was done by a collaborative study group and published several years ago. Lurain wrote this paper. We found that the strain variation was scattered throughout UL97, and some of them were located in these places that were somewhat close to locations of known resistance mutations. So now we have to go back and fill in the marker transfer work, which shows that the H469Y and the B605E, in fact, do not confer resistance.

This marker transfer of which I am speaking is a method for validating the significance of particular mutations by taking them out and transferring them into recombinant viruses that have only that mutation engineered into them, in comparison with a baseline virus. That is a way of telling the specific contribution of a single mutation to the drug-resistance picture.

If you look at different clinical isolates you will find that several common mutations account for the great majority of those that are seen in ganciclovir-resistant isolates. If you take all-comers, you will find that the mutations at codons 594, 595, 460 already account for the majority of
Slide 7 (Continued)

CMV UL97 Kinase Mutations

them. However, many others of lesser frequency are found that fit this guideline of the 590-607 range.

I highlight here the mutation C592G, which appears in the two lists. The one on the left is of all resistant isolates, and on the right-hand side are the ones that were found in those that came from patients who received oral ganciclovir, which gives you questionable drug levels and seems to elicit mutations that confer relatively lower levels of drug resistance, such as the C592G. This particular mutation confers only about a 3- to 4-fold elevation of the ganciclovir IC50. I think it is preferentially selected because perhaps it has the least impact on viral fitness. And so with oral ganciclovir, where you do not have much drug around, the virus just mutates the least that is needed to become resistant.

Slide 8
CMV DNA Polymerase Mutations and Associated Phenotypes

Now in CMV DNA polymerase, the gene mutation story is now almost too complicated to describe briefly because the compilation of mutations now exceeds well over a dozen, if not a couple of dozen. To the extent that one can generalize, it is that the mutations that cluster in this exonuclease region, exo-1, 2, 3, and which overlaps some of the catalytic region of this pol gene, that these mutations tend to confer a combined ganciclovir-cidofovir resistance. Whereas a second large group of mutations that seems to congregate between regions 2 and 3 and especially around region 3, that these tend to confer foscarnet resistance with a certain element of ganciclovir cross-resistance, particularly in region 3.

In region 3 you have this uncomfortable situation where you have mutations that confer foscarnet resistance but also a low-grade or borderline ganciclovir resistance and sometimes a trace of cidofovir resistance as well.

In pol, if you have a mutation in that gene and it is resistant to ganciclovir, you had better assume that cidofovir is not of any use. But in the case of foscarnet, the mutations are largely distinct.
Evolution of Resistance Mutations

After initial exposure of CMV to ganciclovir, usually you see UL97 mutations first. This happens over 90% of the time, which makes it practical and feasible when you are screening for ganciclovir resistance to do genotypic studies only of the UL97 gene. You will miss very few cases of genotypic resistance by checking out only that gene. Later, if you expose a patient heavily to ganciclovir, additional mutations may pile on in the DNA polymerase gene. When that happens, you end up with high-level ganciclovir resistance greater than 30-fold and, as I mentioned, cidofovir cross-resistance.

We do not normally expose patients to foscarnet before ganciclovir. In the limited number of cases where this has happened, usually other DNA polymerase mutations are selected that have limited or no ganciclovir-cidofovir cross-resistance. They happen in positions such as 700, 715, 756, and such.

Foscarnet is the usual second-line drug after ganciclovir resistance develops. However, because of the complicated interplay of these mutations, single or multiple pol mutations are known that confer multiple drug resistance and that is in the end, because all of the current CMV drugs target the viral DNA polymerase gene.

Frequency of GCV Resistance

I can skip over this fairly quickly because Dr. Razonable went over this information, that is, the frequency of ganciclovir resistance in the transplant setting. I tossed in some data from the AIDS population. This figure of 20% going down to 5% represents studies done in different time periods. These studies were done, largely coordinated by Dr. Jabs at Hopkins. Back in the early 1990s they had determined the rate of 20% ganciclovir resistance after a year of treatment for CMV retinitis, but more recently it has now been down to 5% because in the era of HAART, patients are doing much better in general with their CMV disease.
In the solid organ transplant setting, almost always D+R-, and the exact statistics will vary a little from center and center. Some centers that have contributed quite a bit of data include the group in Montreal, the group in Seattle; Lurain had one that was the combination of a Cleveland and Chicago cohort, and so forth.

The data shown here are compatible with what you have heard earlier this evening.

**Slide 11**

**Treatment of GCVr CMV**

The treatment of ganciclovir-resistant CMV, again, foscarnet is a standard treatment. However, the toxicity is high, as has been amply mentioned already. Cidofovir I find is doubtful. It has not been subjected to any kind of controlled trial that shows it to be useful in this population, and beware the problem of cross-resistance based on a *pol* mutation.

Then we have these immunomodulators with anti-CMV activity. These mTOR inhibitors, sirolimus and related drugs. Leflunomide. These immunomodulators that have this activity may have an adjunctive role. They are not FDA-approved and, as we will see, these drugs can only do so much. They may knock down viral replication and cell culture by 50%, 60%, but not 90% or 100%.

Then we have the experimental CMV drugs that we are discussing as well. Maribavir. I will say a little bit about the resistance picture with regard to this new drug.

**Slide 12**

**Resistance – Lung Transplant**

As an introduction to the rest of this, let me just show you this case that we are managing right now as an ongoing case at OHSU [Oregon Health & Science University]. It shows two strategies that have been used to manage resistant virus. So you see later on the left-hand side of this slide, the traditional strategy of ganciclovir resistance emerges and you use foscarnet. And then later on, this possible combination of ganciclovir and foscarnet.
Slide 12 (Continued)

Resistance – Lung Transplant

Here we have a lung transplant patient, and we have heard that that is the highest risk group, particularly D+R\(^-\) minus lung transplant. These patients have the most frequent evolution of resistance, and indeed it happens in this case. The patient gets ganciclovir IV prophylaxis and then somewhere during this course develops rising viral loads and a UL97 mutation that is classic for ganciclovir resistance.

The patient is switched to foscarnet, and you see here a gratifying decrease in loads. The patient had only a fever leukopenia syndrome, no disease. Then after a period of foscarnet, we run into problems: toxicity, renal dysfunction. And so after awhile, the patient was, because of this good control of the viral load, switched to cidofovir. Here is another case where cidofovir did not do its job, and you have rising loads while on the cidofovir. And you have this mutation, A834P, which it turns out confers foscarnet resistance as well as a degree of ganciclovir and cidofovir resistance. So you have here a single pol mutation that confers some resistance to all three drugs.

Here was where it was decided to pull out, representative of the various categories of management I alluded to in the previous slide, and that is the combination of ganciclovir and foscarnet, both pushed to the full therapeutic doses. Of course, in this case they had to be adjusted for renal function. And then also added sirolimus, which has some inherent anti-CMV effect. The combination of all three apparently was successful in bringing down the viral load, but not to 0.

Several months beyond the timeline of this slide, we have a patient who has a persistently low viral load of several thousand copies per milliliter in plasma. We do not quite know what to do with it because the patient remains asymptomatic.

The other message from this slide is that despite severe problems with genotypic drug resistance, it is certainly not the case that every patient with resistant CMV is headed toward death or severe symptomatic disease. There will be cases like this, where you will have resistance in kind of a smoldering situation.
**Slide 13**  
**GCV-FOS Combination Treatment**

People have often asked about ganciclovir-foscarnet combination treatment. Is this synergistic? As far as I am concerned, no. This combination is not synergistic. It may be additive. I tried to do the checkerboard assays and those classic things that you do in the laboratory to determine synergy, and I observed merely an additive effect.

The other way is to ask, is there clinical evidence of synergy. That was attempted to be studied several years ago by Griffith’s group again in London. That was that can we compare full-dose ganciclovir vs half-dose ganciclovir and half-dose foscarnet. This is not in the resistant setting whatsoever. It is just to test the antiviral efficacy of a full dose of one vs a combination half-dose. Because if these are synergistic, well, maybe half of one and half of the other will give you a better antiviral effect. And, no, the answer is it is worse. So this is clinical evidence of no synergy.

However, if you have a ganciclovir-resistant situation, you may still have a benefit from trying to push both drugs to the maximum tolerated. That is probably an okay strategy, as we showed in the previous slide. However, running into toxicity problems is practically routine, as we encountered in our patient as well who developed renal failure after a period and the foscarnet had to be stopped.

So it is worth considering this combination approach, but it is far from satisfactory overall.

**Slide 14**  
**Host Factor Treatment**

There are host factor treatments that have been talked about: sirolimus, roscovitine. These various kinase inhibitors have a measurable *in vitro* anti-CMV effect. In fact, it is even mentioned that patients who receive sirolimus immunosuppression have statistically lower odds of having CMV disease. When we check out these agents in the laboratory, we find that with these drugs it is easy to reduce CMV replication to the tune of 50%, 60%, even 70%, but not beyond that. So mostly adjunctive, and their use is still unapproved.
I think the same story applies to drugs like leflunomide, artesunate, and so forth. These are things that modulate the physiologic condition of the cell, and one day they might be a combination treatment with a main-line antiviral drug.

**Slide 15**

**Experimental Drug: Maribavir**

Again, this is totally reminder material from maribavir. It has all been discussed. It is a new drug that is a UL97 kinase inhibitor. Dr. Michael Boeckh went through the details of all of the Phase I and Phase II trial results.

**Slide 16**

**Maribavir – Antiviral Properties**

Antiviral properties. Maribavir is a selective and potent inhibitor of UL97. Cellular factors affect the antiviral activity, which means that the cell type that you do the assay in affects the measured IC50 of the drug. Some cellular kinase inhibitors, including sirolimus, enhance the perceived activity of maribavir in the *in vitro* assays. And, who knows, that may have a clinical role one day.

There appear to be strain differences in sensitivity to maribavir. Little information is available so far, but needs to be studied.

In terms of the relationship to existing drugs, it is important to realize that because maribavir shuts off UL97 it is also predicted to shut off the phosphorylation of ganciclovir that is necessary for its antiviral effect; therefore, it is predicted to antagonize UL97 and ganciclovir. We do have some laboratory data that support that.

The resistance of maribavir is being explored currently only in cell culture. We do not have any data pertaining to human use of mutations that have been selected. However, maribavir resistance does occur as a consequence of UL97 mutations as well as of UL27 mutations. UL97
mutations in certain locations confer medium- to very high level maribavir resistance, whereas UL27 mutations confer only low-level resistance of uncertain clinical significance.

Importantly, no cross-resistance has ever been observed so far with a reasonable number of isolates that are resistant to one or more of these drugs, and we are not yet seeing any problem at least with cross-resistance.

**Slide 17**

**CMV UL97 Kinase Mutations**

In terms of the mapping of the mutations, fortuitously now, we have this situation where maribavir resistance mutations map to a distinctly different part of UL97 than the ganciclovir resistance mutations. The closest they come together is in this 460 region or between 411 and 460. And so we made some mutants in our laboratory and checked out how these behave with regard to maribavir and ganciclovir. We find that these mutations listed here for maribavir resistance confer only maribavir resistance.

In color, the IC50 ratio. You can take an IC50 ratio of greater than 2, 3, 4 as being resistant. And so you see that for ganciclovir none of these approaches even 2, and so it remains sensitive.

The classic ganciclovir resistance mutation, 460V, gives you an 8-fold resistance to ganciclovir and is, in fact, hypersensitive to maribavir. Very interesting.

As a result, despite the apparent proximity of these mutations, we are not actually seeing any problem with cross-resistance so far. It only stands to reason that one of them is a kinase inhibitor and the other is a kinase substrate. So let us hope that these mutations remain apart in clinical experience. We are still waiting to study any isolates that come out of our clinical trials.

**Slide 18**

**CMV Resistance – Summary**

In summary, the risk factors for CMV drug resistance, we have gone over. If there is increasing viral load during prolonged treatment, confirm with genotypic testing if possible, based on
known mutation patterns. Foscarnet is our usual alternative drug; therefore, the ganciclovir-foscarnet combination is possible but may be toxic. Immunomodulation, optimize that, try and cut it to the least amount that you can use and that may be certain drugs that are better than others for immunomodulation. Then we have our new drug that we are hoping gives us a new way of avoiding the drug-resistance problems that we have had with our traditional therapies.

Thank you.