

## Annual International Conference

# 39<sup>th</sup>

# Orthomolecular Medicine Today

## Early Cancer Detection

By

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### Preamble

Prof. Dan Burke was invited to speak on “Secondary Plant Metabolites and Cytochrome P450 Enzymes in Cellular Regulation”. Shortly before the conference began Dan had to enter hospital for back surgery. Given this unforeseen situation I was asked to take his place. I took this opportunity to introduce our work on early Cancer Detection. The following is the script from this presentation.

### Introduction

Dan Burke put together a great presentation on secondary plant metabolites and cytochrome p450 enzymes. Unfortunately Dan is currently in hospital awaiting back surgery and he asked that I give his talk. Dan and our English colleagues have embarked on a program of research investigating prostate cancer and breast cancer, specifically looking at the metabolism of secondary plant metabolites by CYP17 and CYP19 along with some of the preceding metabolism that takes place with other members of the CYP family. Subsequent to Dan preparing his

presentation we received some data on some recent work that we have been conducting that I find really exciting so I have decided to abandon Dan’s presentation in favour of filling you in on this current work and the results that we have just obtained.

What I would like to do is to introduce some work that we have been doing over the last couple of years. The work builds on the prior work of this team so I will start with a brief summary and a brief history to provide a context and then get on with telling you about the work we are currently doing.

## Summary

Many of you will be familiar with salvestrols – there is an article in the current issue of JOM. The basic story line is that salvestrols are food-based, secondary plant metabolites that have a specific functional relationship to a cytochrome p450 enzyme called CYP1B1. CYP1B1 is a unique cytochrome p450 in that it doesn't occur in healthy tissue – it only occurs in cancer cells. It is also unique in that it is a universal cancer marker occurring in every cancer tested.

What the team discovered is that salvestrols work as natural prodrugs. They are exceptionally stable and non-reactive compounds that bind with CYP1B1. The metabolism that results from this binding produces a metabolite that induces apoptosis in the cancer cell. This is the basic discovery of the research team in Leicester, England.

## History

I will now give you a bit of history as to how we got to the work that I want to introduce. The team has two key scientists: Prof. Gerry Potter and Prof. Dan Burke. Gerry is a medicinal chemist and cancer drug designer. Dan is an emeritus professor of pharmaceutical metabolism, and an expert in cytochrome p450 enzymes and toxicology.

Close to 20 years ago Gerry got his first taste of designing prodrugs. The London based Institute of Cancer Research group that he was with were working on prostate cancer and had a great interest in CYP17. Gerry examined CYP17 in detail, worked out

its metabolic action and designed a prodrug that targeted CYP17 to perform a terminal inhibition. The drug is called abiraterone acetate. The clinical trials of this prodrug have been exceptionally successful. The company that has taken abiraterone through clinical trials, Cougar Biotechnologies, just sold for \$1billion to Johnson and Johnson on the strength of this drug. Given this drug's performance Gerry has been named cancer researcher of the year by a UK prostate cancer foundation and asked if he would be their President.

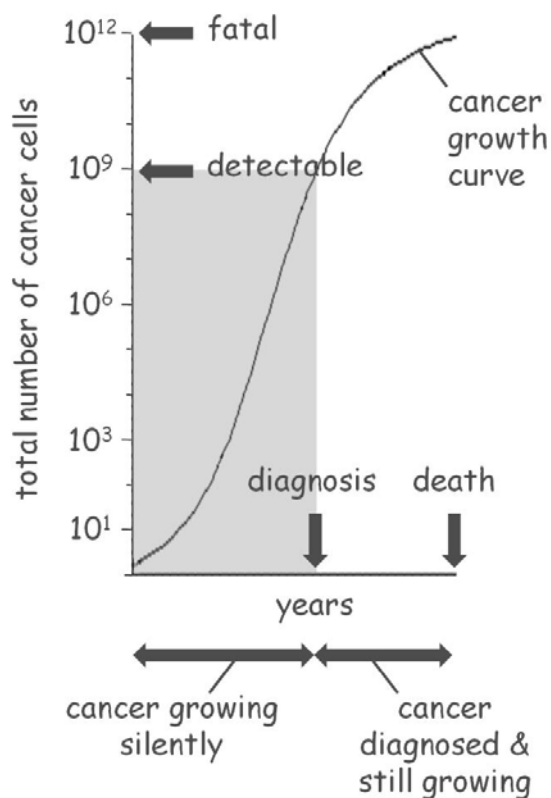
Around the same time as Gerry was designing abiraterone, Dan Burke began his investigation of a new p450 CYP1B1. He found it in soft tissue sarcomas. He later found it in cancers of the breast and subsequently found it a variety of cancers including cancers of the breast, colon, lung, oesophagus, skin, lymph node, brain, and testis with no detectable presence in healthy tissue. Basically Dan and his graduate students identified CYP1B1 as a universal cancer marker.

Dan and Gerry met some years after these two pieces of work. When Dan told Gerry of his exciting new enzyme Gerry immediately drew upon his experience designing abiraterone for cYP17 and started looking at how he could design a drug to target it. Within two weeks of hearing of this enzyme Gerry designed a prodrug that was metabolised by CYP1B1 to produce a metabolite that killed the cancer. Cell line results with this drug were remarkable. The drug was perfected over the next nine months, patented, sold off and the long road to clinical trials began.

During this time Gerry believed that CYP1B1 was a natural rescue mechanism and if this was true there had to be analogues of his prodrug in food. Gerry led a search for these analogues, found quite a few of them and the subsequent research program surrounding salvestrols was born. Incidentally Gerry's prodrug has an unbelievably high selectivity for cancer cells – over the past few years natural analogues of this prodrug have been found that have vastly higher selectivity than the prodrug that Gerry designed.

and  $10^9$  cells (if you look at the nail on your little finger, half that size is between  $10^8$  and  $10^9$  cells – roughly the size of a pea) – once cancer reaches  $10^{12}$  cells (about a litre of cells) you're dead. By the time modern technology can tell you that you have this disease the disease has silently grown through about 75% of its life. Danny wrote a really nice paper on this subject and I have copies for anyone that is interested.

### The Need For New Clinical Tools



The other side of the problem is that once you are told that you have this disease there are really poor tools, for most of the cancers, for monitoring disease progression and treatment efficacy.

The graph has implications for people that start to think about cancer prevention. They, of course, assume that they are free of the disease. They may consult a physician and take advice on cancer prevention. But this advice is likely to also assume that they are free of the disease, however, they may lie anywhere on that curve below the level of detection. If they are already up that curve preventive doses are going to slow the rate of growth but not keep the cancer from breaking through into the detectable range.

The graph also has implications for people that have journeyed through this disease to the point that their physician has told them that they are 'all clear'. This statement may simply mean that their disease is again below the level of detection.

As the research developed we kept having conversations about the need for better clinical tools. The difficulty was twofold. Current technology can only detect cancer once the cancer has achieved between  $10^8$

All in all it amounts to a pretty miserable picture.

Wouldn't it be great if we had a simple blood test that could be used to screen for any of the cancers, with a sensitivity that could pick up the presence of the disease long before it has reached  $10^8$  and  $10^9$  cells? Think about how much easier it would be to assist these people back to good health. And wouldn't it be nice if a simple blood test could be used to monitor any of the cancers with a level of accuracy that would readily tell if a treatment is working or not and whether a dose is high enough? A blood test that is as applicable and accurate with pancreatic cancer as it is with breast cancer – a blood test that is as applicable and accurate with adrenal cancer as it is with prostate cancer. Tools such as these could make life a lot easier for clinicians and patients alike.

### **Development of Clinical Tools For Early Cancer Detection and Monitoring.**

The need for new clinical tools is obvious. One of the enormous implications of the prior work of Gerry and Dan is that it sets the stage for the realisation of blood tests such as those that I just described.

We started to look at what we had to work with. We had great expertise on CYP enzymes, we had great expertise on secondary plant metabolites and their metabolism by CYP enzymes. Specifically, we had CYP1B1, a universal cancer marker and salvestrols, natural prodrugs, which in this context amounts to things to look for in bodily fluids. Given the salvestrol – CYP1B1 mechanism there should be things that we could look for that would tell us about the presence and state of this disease. Basically we could use our understanding of this

metabolic relationship to report back to us on the disease itself.

We took the decision to utilise this knowhow and develop clinical tools for the early detection of cancer, and treatment efficacy. One thing that we have learned so far on this project is that it is a really good idea to have people on your research team that don't understand enough to know that it can't be done.

In considering the problem we decided that we had one of two directions to take. The obvious first route was to develop a method for detecting and measuring the presence of cyp1b1 itself. Since CYP1B1 is an intrinsic component of cancer cells if we could detect and measure it in blood or urine we would have a direct measure of the disease itself. The second, and much less obvious approach, to develop a method for detecting and measuring the metabolic output of cyp1b1. If we could find a strong metabolic output of CYP1B1, detect and measure it we would have another direct measure of this disease. So we decided to pursue both – two universal tests for cancer.

### **Proteomic Approach:**

In pursuing the detection and measurement of cyp1b1 itself we knew that the job would be made much easier if we had antibody. We wanted an antibody to an amino acid string that was 100% specific to cyp1b1, covering the wild form and the major polymorphs and not found in any bacteria and one that didn't have major cleavage sites running through the middle of it. These criteria ruled out all of the antibodies that are currently available for cyp1b1. We

performed an exhaustive search and identified a set of peptides that met our criteria and embarked on raising antibodies.

CYP1B1 is a very difficult enzyme to raise antibodies for that have a strong affinity for the peptide of interest because *cyp1b1* is present in so many life forms in identical form or near identical form to that found in humans. However, we did manage to raise an antibody to a specific *cyp1b1* peptide and worked on affinity enhancement until we had something useable.

Our first notion was to see if we could detect and measure *cyp1b1* in human tumour samples. Seemed like a good idea at the time – where else are we going to find *cyp1b1* in abundance?

We spent about a year working on sample preparation methods and testing samples using some of the world's most sophisticated mass spectrometry equipment. We spiked the matrix with *cyp1b1* from recombinant sources and managed to recover the recombinant material but never managed to detect the native *cyp1b1*. This caused us some considerable concern because the wisdom of the day dictated that if we couldn't manage to detect and measure *cyp1b1* in tumour samples, where it would be plentiful, we would never be able to detect and measure it in blood or urine. Given that we were able to detect and measure the recombinant CYP from the tumour matrix we knew that we had a sample preparation and extraction problem – we were either not freeing the enzyme from the surrounding material or we were destroying the enzyme with our preparation method.

In light of this we decided to abandon our search for CYP1B1 in tissue and focus on detecting it in blood. This decision flew in the face of conventional wisdom but our thought was that if we were ever going to have a viable diagnostic and monitoring tool it had to work on blood or urine samples so if we were going to pound our heads against a wall it might as well be the wall we needed to get to. It isn't really as crazy as it initially sounds – even though everyone told us that we were crazy. When working with blood you don't need some of the sample prep steps that you would use with tissue because you don't have as much intact material to deal with – you are already working with fragments.

So we embarked on trying to find our CYP1B1 peptide in blood. AND we ended up with the same results as we found for tissue. We spiked recombinant CYP1B1 into blood and managed to recover it but were unable to recover native CYP1B1 amidst a chorus of 'I told you so' UNTIL one member of the team came up with the bright idea of starting with more blood! We increased the initial sample size and detected and measured our native peptide.

### **Proteomic Results**

The naturally present CYP1B1 peptide was successfully detected using antibody-affinity capture in both 20  $\mu$ l and 200  $\mu$ l digests of cancer patient plasma. The amount of natural CYP1B1 in this sample can be estimated to be  $\sim$ 200 amol/ $\mu$ l of plasma.

This result is very recently achieved but we have had an opportunity to replicate with 5 additional samples.

Sample	Amount of CYP1B1 (amol/ -l of plasma)
2 ml tube labeled 22/04/2009	12.5
2 ml tube labeled 23/04/2009	2.0
2 ml tube labeled 24/04/2009	9.4
2 ml tube labeled 25/04/2009	9.2
1.5 ml brown tube non-labeled	4.9

Lower levels of peptide were found in these samples with the amount of natural CYP1B1 ranging from 2 to 12.5 amol/  $\mu$ l of plasma. (MRM analysis was performed by injecting 15  $\mu$ l on an AB/MDS Sciex 4000 triple quadrupole mass spectrometer equipped with a Nanoflow Eksigent NanoLC-1Dplus HPLC, 5 x 0.3 mm C18 Pepmap (5  $\mu$ m particles) trap column, and a 75  $\mu$ m x 150 mm Magic C18AQ (5  $\mu$ m particles, 100 Å pore size) analytical column using 30 min. analyses.)

### Proteomic Summary

At this point we have a sample preparation method and an antibody (an assay) that is able to directly detect and measure cancer though detection of CYP1B1 in plasma. When we find our peptide in your blood with this assay you have cancer – there are no false positives – you have cancer.

We have been working on research machines but we have now identified a mass spec machine that is designed for use in clinical laboratories as a platform and we will be in Germany to work out an arrangement with them this coming week.

We now have a variety of method enhancement experiments, stability experiments, validation experiments and method transfer experiments to conduct but at least at this point we know it is present in the blood, we can find it and we can measure it.

What I really like about this approach is that it will be simple and convenient for the person getting tested. Simply put out your arm for a sample collection like any other blood test. What I also like about this approach is that it is a direct detection and measurement of the cancer itself and it is applicable to pancreatic cancer as it is to breast cancer – it is applicable to all the cancers. The other thing that I like about this test is that we are operating at an exceptionally high level of sensitivity and we have good reason to believe that we can increase the level of sensitivity from here.

### Metabolite Approach

We know the various substrates of CYP1B1, that is, we know what it metabolises and in particular we know a lot about the salvestrols that it metabolises. So what happens when we ingest salvestrols?

In our food salvestrols come in two forms: as a glycoside and as an aglycone – in food about 80% as glycosides and 20% aglycones – in capsules 100% aglycones. When we ingest the glycoside the plant sugar is cleaved off and replaced with a human sugar. When we ingest the aglycone a human sugar is attached. This of course assumes that everything is working properly to perform this function. The new glycoside is then transported and upon reaching cancer cells the human sugar is cleaved off leaving the aglycone at the cancer site. This step is performed by Beta Glucuronidase. The aglycone then binds with CYP1B1 and is metabolized. The metabolite induces apoptosis spilling the contents of the cancer cell, including CYP1B1 peptides and metabolites into the surrounding space.



What all this means for blood test development is that the interaction of CYP1B1 and salvestrols provides us with a variety of measurable aspects of this process that can provide us with insights into the presence of disease as certain of these aspects can only be present if the disease is present and metabolism has taken place.

What we did initially is go through our list of salvestrols looking for metabolites that were abundantly produced through CYP1B1 metabolism and not found in a typical diet. From a candidate list one metabolite was chosen.

We looked to see if we could find the aglycone in blood and urine – initially used predicted structures and then using synthesized standards we were able to reliably detect and measure the aglycone in both blood and urine. We then performed a pharmacokinetic study using healthy volunteers to determine when salvestrols reach peak concentration in the blood – three hours after ingestion. We identified the aglycone spike resulting from salvestrol using hplc. Prior to hplc analysis the samples were prepared and Beta Glucuronidase was used to remove the sugar from the glycoside.

Following this we decided to take a look and see if we could find a difference between healthy volunteers and those with advanced cancers. We administered 1 gram of a specific salvestrol to each individual, waited 3 hours and drew their blood. We also had each individual do a 24 hour urine collection. As expected, with healthy volunteers we found no metabolite – we

simply recovered the substrate in blood and urine. With diseased volunteers the situation was very different. We found a very clear spike on the hplc where we predicted that the metabolite should come off the column. Some of these individuals had really very advanced disease and with these individuals we found absolutely no aglycone and no glycoside – just metabolite. When we analysed their urine we also found no aglycone. The entire gram of substrate seemed to have been used up. With other cancer patients we found small amounts of aglycone along with large metabolite spikes. What this tells us is that the ratio of metabolite to aglycone may be of much greater clinical value than the metabolite alone – time will tell. We performed these tests with individuals representing a fairly broad array of common cancers: breast, stomach, kidney, prostate, etc., and an array of stages of cancer but skewed towards more advanced cancers. Metabolite spikes were found for all as one would expect given that we are looking at the metabolic output of a universal cancer marker.

### **Metabolite Summary**

So at this point we have a sample preparation method that allows us to detect the aglycone and the metabolite in blood or urine using hplc. We find clear separations between the outputs obtained from healthy volunteers as compared to diseased volunteers. Like the proteomic approach when we find this metabolite in your blood you have cancer.

What I really like about this approach is that it uses natural products as diagnostics. We are getting the metabolism of a natural

product to report on the presence and state of disease. Another nice feature of this approach is that we can build the signal by the amount of substrate that we administer. Another thing that I really like about this approach is that it not only tells us that CYP1B1 is present, that is that cancer is present, it can tell us that the enzyme is functioning fine.

What I also like about this approach is that we have been drawing blood at 3 hours, the time of peak concentration for the substrate. We are about to embark on a pharmacokinetic study to determine peak concentration of the metabolite. Once we are able to draw blood coincident with peak concentration of the metabolite we will be able to pick up the presence of cancer much earlier. Like the proteomic test the metabolite test is universally applicable.

### **So where are we?**

A present we have two different assays for detecting and measuring the presence and amount of cancer. Both operate independent of any apriori notions about type of cancer that may be present.

The huge strength of these approaches is that they can be used with all cancers – they are two universal cancer tests that can ultimately be used for diagnosis and monitoring across all of the cancers. The downside of this is that we will need to validate both approaches on each and every cancer which means that we are going to be very poor long before we complete this task.

Up 'till now everyone on the team has had their pet blood test – either the metabolite

test or the proteomic test. However, from the onset there have been good arguments for seeing both of these approaches through to completion because they provide differing set of strengths and weaknesses and when we combine them we can potentially provide much more clinical assistance that we could with results from either one.

For example, let's say that we have two 36 year old females, very similar in family history, medical history, etc., and both have a 2 cm cancerous lump in one of their breasts. Their physician decides to run the metabolite test. With one of the women a large metabolite spike is found with no aglycone and no glycoside. With the other woman we find a medium sized spike of metabolite and small spikes of aglycone and glycoside. What is going on? With just the metabolite test we might conclude that woman number 1 has fully functioning CYP1B1 that is making full use of the substrate while woman number 2 may have competing substrates in her body that are inhibiting the functioning of CYP1B1 – for example she may have been using some household paints that contain chemical anti-fungal agents or perhaps she had the furnace duct work recently cleaned and the cleaners used chemical anti-fungal agents to retard any fungal build up or perhaps she takes daily walks along the perimeter of a golf course that uses a great deal of anti-fungal spraying. We may also conclude that woman number 1 may have an additional undetected tumour mass. If we now run the proteomic test we can help to determine what in fact is going on for these two women. Let's say that we run the proteomic test and find a larger spike of peptide for



woman number 1 than woman number 2. This result would tell us that there may be no difference at all between the functioning of CYP1B1 for these two women but rather confirm that woman number 1 has another, undetected tumour mass and this is accounting for the higher results. The attending clinician can then embark on a search for the whereabouts of this second tumour mass.

### **Where do we want to get to?**

We want to keep pushing the limits of detection until we are picking this disease up at disease onset.

We want to be able to pick this disease up at points where simple dietary and lifestyle change can turn the disease around. That is one of our goals.

We also want to push the sensitivity of these tests to the point where we can tell whether or not a treatment is working within 24 or 48 hours after commencing

treatment. This will be exceptionally beneficial to people pursuing conventional chemotherapy as it could save them from weeks of toxic exposure.

We also want to be able to utilise these tests to individualise treatment plans for cancer sufferers.

We want to push the sensitivity of these tests to the point that we can pick up disease recurrence at a point where dietary and lifestyle change can turn the disease around.

Along the way to reaching these goals and providing the evidence we are able to add value to those volunteers that contribute their blood through advising their physicians of the results.

I am sorry that Dan Burke was unable to attend. Hopefully Dan will be able to make it to your next conference. Thank you for your attention.

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