Selected Proceedings of ALDAcon 2004
"Green Mountain Odyssey"

The Merlin-Pak Connection Revealed: The Turning Point in the Development of Therapeutics for NF?

Hiroshi Maruta, Ph.D.

I am a very basic scientist. Actually, I have never spoken to so-called lay people, so I'm a little bit scared. I'll try my best to make my message clear in the simplest terms. My original research has been focused on the ras cancers which are caused by a particular gene called ras. This causes more than 90% of pancreatic and more than 50% of colon cancers. That is why ras cancers have been my original focus, but during our research we noticed that there is a very close relationship between NF, neurofibromatosis (both type 1 and type 2), and ras cancers.

About a decade ago, as soon as both NF1 and NF2 genes were cloned or discovered, we started working on NF. By the end of this talk you'll see that either NF1 or NF2 is not isolated from other cancers, but they share many common things. In fact at the molecular level, more than 50% of all cancers are closely related to NF, type 1 and type 2. That's the reason why we think the drugs which block a particular enzyme called pak could be potentially the first cure for NF, as well as many other cancers. My talk today will focus on the relationship between ras cancers and NF and my major focus is on a particular enzyme called pak.

I think you are much more familiar than me about what NF2 is. So this slide shows only symbolically one of the typical NF2 diseases called meningiomas. Before I discuss the details of how ras cancers and NF are related, I'd like to give you a very short introduction, in particular the current situation of NF2 treatment. As you know, more than one and a half million people on this planet are suffering from neurofibromatosis (NF), in either type 1 or type 2; however, the status of NF treatment is very far from satisfactory. One of the reasons is that treatment is limited to only surgery and radiotherapy. However, for complete treatment of NF, these two types of treatment are not complete.

There are two reasons. One reason is that neurofibromatosis is highly metastatic so that although the original tumor is developed in brain or along the spinal cord, eventually the tumor spreads over the whole body so that the local treatment by surgery or radiotherapy wouldn't be sufficient for a complete cure. The other reason is that the sites where the tumor grows are in the brain and on the spinal cord so that it would be very difficult to remove or destroy the tumors without affecting the surrounding healthy

nerve. However, no therapeutics have been developed for the complete cure of NF as of yet.

One question you might ask is, why conventional anti-cancer drugs are not used for NF1 or NF2. Well, there is a reason. Actually, these conventional therapeutics are not effective in either NF1 or NF2, simply because these conventional drugs were developed to target mainly the fast growing malignant tumors. Unfortunately, as you know, the majority of neurofibromatosis (NF tumors) are not malignant and only 10% or less are so-called cancer or malignant. Also the growth of these NF tumors is very slow. The conventional drugs targeting on fast-growing tumors, like leukemia, work only on tumors that grow very fast, but never work on the very slow tumors. Also, these chemotherapeutics have severe side effects on some normal cells such as hairs and bone marrow cells, which also grow very fast. That's why we, not only my own group, but also many other cancer research groups, decided to develop a new type of chemical compounds which block the specific signaling pathways essential for tumor growth. That's one of the reasons why we started with ras, because ras is probably the best known oncogenic or cancerous gene product, and biochemistry of signaling pathways down-stream of ras has been most comprehensively studied during last two decades. In the end we found that both NF1 and NF2 share a common signal with ras cancers and many other major cancers. So first, let me try to tell you how ras works.

Ras is a signal transducing protein, and there are two stages. One is the active form, which binds a certain nucleotide called gtp. In the normal cells, ras cycles between the inactive form and active form. Through two other proteins, “a” converting ras from the active form to inactive form (gdp-bound form), or “b” only upon the stimulation of cells by growth factors, reactivating the ras. Normal ras stays only transiently in the active form. That's why normal cells will never get malignant. However, when ras gene is mutated permanently, it is kept frozen in this active form, and keeps sending a signal continuously to its downstream effectors. Then that causes tumors.

Among several effectors which are activated by ras are pi-3 kinase, raf and tiam. But you don't have to remember those names. By this slide I'd like to show how ras cancers and NF1 and NF2 cross. First of all, I'd like to show the way how ras activates other proteins simply called a, b, and c. A activates b, and b in turn activates c, through this signal cascade. In the end the key enzyme pak is activated that we are working on. So ras activates pak through these signal transducers (a, b, and c). We know that for ras to induce tumors requires this enzyme pak. We're working on pak for almost 30 years so that we know the every property of this pak, and we are on the best position to develop its inhibitors.

NF1, type 1 neurofibromatosis, is caused by dysfunction or loss of a gene called NF1. The gene product for NF1 happens to be an attenuator of normal ras. So when NF1 gene is missing, the normal ras becomes abnormally activated, then eventually through this a, b, c pathway, this pak is activated. So in terms of the signal, the type 1 NF behaves in the same way as ras cancers such as pancreatic and colon cancers.

Several years ago Jeff Field's group at University of Pennsylvania showed that this enzyme is essential for the type 1 neurofibromatosis. It means that if we have had a specific inhibitor for pak, we could have treated at least type 1 NF since then, but until very recently nobody has developed any anti-pak chemical compound. That is why there is so far no treatment or chemotherapeutics to treat NF1.

The type 2 NF is caused by a loss of a gene called NF2, or its mutation causing a loss of its tumor suppressor function. But NF1 and NF2 gene products are structurally so different, so it was very difficult for us to find the exact function of NF2 gene product that we call "merlin" (named after a magician in the legend of King Arthur). As soon as the NF2 gene was discovered by Jim Gusella's group at MGH and a French group in Paris, we decided to test whether NF2/merlin is a tumor suppressor. So putting this gene in a ras cancer, we watched what would happen. As expected,the malignant transformation is reversed, so we know that both NF2 and NF1 genes block somewhere in this oncogenic ras pathway. However, it has been difficult to sort out what the primary target of this merlin is (it turns out to bind several proteins). Eventually two years ago we found that the merlin acts as an inhibitor in pak. In other words, when this NF2 gene is missing, pak is abnormally activated, just like the loss of NF1 or mutation of ras. So actually the type 1 and type 2 of neurofibromatosis are in the same family as the ras cancers. Furthermore I will show you in the next slide that loss of NF2 actually activates pak.

We have two types of mesothelioma cancer cells which are derived from lung cancers caused by asbestos, but one normally expresses NF2 gene, while the other is NF2-deficient. Then we compared the pak activity between these two cell lines, using a ras cancer as a positive control which has a very high pak activity compared with normal cells.

The pak activity in normal cells is only one-tenth of that in ras cancer. When merlin is missing in cells, pak activity becomes very high, quite similar to ras cancer. But in the cells expressing normally NF2, the pak activity remains very low (like normal cells). The next question is whether this pak is actually essential for the growth of NF2 tumors. We have two inhibitors for pak. One is a peptide which blocks the interaction between two proteins, pak and pix. I will not tell you details, but the part of pak has some 18 amino acids stretch. This is required for its interaction with the pix that activates pak. So if we use this peptide/stretch, we have to make it cell-permeable before putting in a medium. Then it blocks this interaction (within cells).

Also we used another inhibitor, a chemical compound called cep-1347. This is also a specific inhibitor for pak. Using these inhibitors, we tested how the growth of NF2-deficient and NF2 positive cancer cells is affected. As you see, both these chemical compound and peptide inhibit the growth of only NF2- deficient cancer cells, having no effect on NF2-positive cells. So this clearly shows that the growth of NF2-deficient cancer cells require pak for their growth. However, these inhibitors have relatively high ic50 (are not so potent). So we tried to find out a much more potent pak inhibitor which can be useful for a clinical trial. In the end we found another chemical compound called fk228.

>> Maybe you should explain ic50, what it means. That would be good for people.

Ic50 is the concentration of a chemical compound which shows 50% inhibition of the growth . The lower ic50 is, the more potent it is. So we are looking for something whose ic50 is very low. As you see, even at 0.1 nm, ten thousand times lower (than ic50 of cep-1347 etc), fk228 can completely block the growth of NF2-deficient cell growth. This is its chemical structure. It is a ring peptide. This chemical compound has been in clinical trials for a variety of tumors or cancers, but not yet for NF tumors. This slide would show how this compound fk228 blocks the pak pathway. This chemical compound does not directly bind pak, but blocks both upstream and downstream of pak. Fk228 was developed originally as an anti-ras cancer drug by a Japanese pharmaceutical company called “Fujisawa”, but until recently nobody knew how it blocks the growth of ras cancers. A few years ago they found that this chemical compound changed the structure of chromatin, a complex of genes, and a subset of genes are activated by this chemical compound. Two of them happen to be the tumor suppressors, gelsolin and p21. This scheme shows how ras activates the cell division through pak. The gelsolin blocks the enzyme “a” right after ras. So in the presence of this chemical compound, pak is no longer activated. That's why this compound blocks the growth of ras cancers.

Also this chemical compound, through another protein called p21, blocks downstream of pak. So this single chemical compound gives a double punch to this oncogenic ras pathway. Eventually we actually showed that this chemical compound reduces the pak activity in ras transformed cells. The control cells show a very high pak kinase activity, but when you treated cells with this drug at this concentration, the pak activity is significantly reduced. So it's now clear that this compound blocks the pak activation. To see if both NF1 and NF2 deficient tumors require pak for their growth, we tested whether this compound has any effect on their growth. As we see, when you treat these two cell lines with this drug, none of them grows. At this concentration (0.1 nm), the inhibition is 100 percent. Then we determined the ic50, that is the concentration of chemical compounds that inhibits the growth by 50%. It turned out to be around 5 pm. Very, very low concentration. We used the cell line h-meso, derived from a lung cancer (mesothelioma) caused by asbestos, as this is the most fast growing among NF-2 deficient cell lines. Most NF tumors grow very slowly. So it is very hard to do any animal experiment with NF tumors.

Using this cell line, we can have two types of xenograft, by transplanting this human cell line in immuno-deficient mice (called nude mice). One is a solid tumor. This one grows very slowly. The other one is ascites which is supposed to grow much faster. To see whether this chemical compound suppresses the growth of NF2-deficient cells, it would take probably a few more months, even using this ascites tumor. The prime reason is that growth of these cell lines are much slower than the major cancers, such as pancreatic and breast cancers.

So meanwhile, we chose a much faster growing human cancer to find out the best condition, so-called minimum effective dose of fk228 that once we know the minimum dose, we can start a clinical trial for NF1 and NF2.

There are two reasons why we chose breast cancers. Firstly, about three-quarters of breast cancers are estrogen-dependent, and estrogen receptor and the key enzyme pak form a vicious cycle. The receptor activates pak and in turn pak activates the receptor. So we thought if you block the pak, then the estrogen-dependent growth of these breast cancers could be inhibited. Also about 30% of breast cancers are developed by mutation of other receptors called erbb1 and erbb2. These receptors are activated by peptide hormones. The receptors eventually activate pak through the ras pathway that I have shown you. So these breast cancers are also probably sensitive to fk228. So we recently started to determine the concentration that fk228 needs to block the growth of the breast cancer. As you see, it is around 5-10 pm, very similar concentration at which the growth of NF1 and NF2 cells is inhibited. So in terms of sensitivity, this breast cancer is very similar to NF1 or NF2 tumor cells. Since this develops a tumor very fast in nude mice, we thought this would be a good model for determining the minimum effective dose of this chemical compound in vivo.

Right now we have a xenograft of this human breast cancer in mice. Since the pharmaceutical company provided us with fk228, we cannot release the detailed data right now (without their permission), but I will tell you that the growth of this breast cancer is almost completely blocked by this drug with a certain dose. In the remaining 10 minutes I'll show you a few other anti-pak drugs which block either upstream or downstream of pak. You have seen that fk228 suppresses not only NF1 and NF2, but also breast cancer, and ras cancer. Also last year it was published that the same compound blocks the growth of prostate cancer, indicating that more than 70% of all human cancers/tumors can be treated by anti-pak inhibitors such as fk228. But as you know, even a miracle drug called gleevec, probably a single chemical compound would not lead to the complete cure, simply because some mutated tumors then begin to show the resistance to this drug. So we like to have multiple drugs which block different target enzymes, but in the same pathway. That is one of the reasons why we developed another inhibitor called ag 879 which blocks theinteraction between pak and etk. The etk is another enzyme which is required for the activation of pak. The pak requires so many proteins for the activation in cells. So if you block this etk, you can block the ras pathway, and thereore ag 879 also could be useful for the treatment of NF. Besides, we are trying to develop a direct pak-specific inhibitor called st-2004 by a specific chemical modification of a non-specific inhibitor called st-2001. The original compound found in a marine sponge is a very potent inhibitor for pak, but inhibits so many other enzymes as well. So it causes even the death of normal cells. So you can't use it as a drug. However, we know how to modify it chemically to reduce these side effects selectively so that its derivative st-2004 blocks only pak in the end. We are making this “magic bullet”. Also a drug company called Bayer, and a biotechnology company called onyx together developed another inhibitor called bay 43-9006. This is specific for another enzyme called raf just downstream of pak. This is now in clinical trials for ras cancers and many other cancers. So this is also potentially useful for the treatment of NF1 and NF2, because it blocks right downstream of pak, and this pathway is actually required for the growth in NF2.

Using just a few last minutes, I will show you how to modify and convert the non-specific pak inhibitor to a potent pak specific inhibitor. Actually, I have mentioned to you that the cep-1347 is a specific inhibitor for pak, but the ic50 is very high, around 1 micro m, and in our common sense we can't use it for so-called in vivo (animal or clinical) experiment, because we have to use a very high dose to see the effect. So we began to focus on the compound st-2001, a product of a marine sponge found in Guam Island. This is a thousand times more potent than cep-1347. The pak-specificity of cep-1347 is based on the unique property of pak that distinguishes itself from other enzymes. The drug-binding domain of the enzyme pak is much bigger than any other enzymes called kinase family. So if you put these bulky side chains, only pak can accept this compound. So although st-2001 itself binds any other enzymes non-specifically, if you put this bulky side chain to this compound, converting this one to st-2004, then it is expected to inhibit only pak, and not any other enzymes, and it is very potent. That is what we are making in collaboration with people in guam island and organic chemists at yale. Because we need a large quantity of st-2001, it takes some time, but hopefully by the end of this year we get this starting compound in a large scale, and then convert it to this pak-specific potent inhibitor.

One of the chemical compounds that works at least in ras cancer in mice is called ag 879. This blocks interaction between pak and etk. It inhibits the growth of ras cancer by only 50%. There is another chemical compound called pp1, which was originally developed by the pharmaceutical company pfizer several years ago. This inhibits another enzyme in the sarc family. Again it alone inhibits the cancer growth only by 50%.

If you combine these two compounds, the growth of ras cancer is almost completely blocked. We are going to do the same experiments with human NF1 and NF2 tumors. If like ras cancers, it is proven to work with NF tumors, we can shift to clinical trials. I have talked about a few chemical compounds which block upstream or downstream of pak, but just before closing my talk, I will show you another way to treat NF, both type 1 and type 2. This is a little complicated. I'll just try to make it simpler. This approach is somehow opposite to the chemical approach.

There is a unique virus called reovirus. This is a deadly virus for mice, but harmless for humans. Most people have probably never heard about it. It became known (several years ago) that the reovirus can destroy only the cancer cells in which the ras is activated. Thus, ras cancers are the major target of this virus. This reovirus does not cause any harm to normal cells. So it's now clinical trial (I think phase 1 or 2), and it shows some promising result.

Recently we found how ras allows this virus to infect the cells. The responsible enzyme that allows the reovirus to infect cancer cells is actually pak, because pak interacts with this and other proteins anyhow. Par in principal, all the cancers, in which the pak is activated, like ras cancers and NF tumors, they're sensitive to this reovirus. So after the treatment with so-called pak inhibitors of NF1/ NF2 tumors, and if we find that some of the tumors become resistant to the anti-pak drugs, we know in those cancers the pak is fully activated, and therefore the tumor is sensitive to this reovirus. So we can treat

those tumors, including NF1 and NF2 tumors, with reovirus. Instead of blocking the pak pathway, using this pak pathway we can actually treat neurofibromatosis.

These are in our hope, and some of the viruses or chemical compounds are now clinical trials. The only thing that we have to show (prior to NF clinical trials) is that these viruses or chemical compounds work at least in mouse NF models. Then we are allowed to shift to the clinical

Trials. That is our current situation. There is a hope for most of NF1 and NF2 people. To close my talk, I'd like to show a few people who have contributed to this work. This is my small group of mine in Melbourne who contributed to the work on pak inhibitors. These two persons (Hong and Thao) contribute to show that pak is essential for ras cancer's growth and also developing the pak inhibitors. This lady (Yumiko) actually showed that NF2 is an inhibitor for pak and also showed that the pak inhibitors actually block the growth of both NF1 and NF2 tumors. Also these (gentlemen) are chemists at Yale who synthesized the pak inhibitor (cep-1347). Now they are also trying to convert the marine compound to a much more potent pak-specific inhibitor, st-2004. Several other people also helped our work. First of all, Alex Levitzki's group provided us with ag 879. Also our neighbors, Jonathan Baell provided us with a water-soluble derivative of ag 879. Fk228,

The most potent pak inhibitor, was provided by Fujisawa pharmaceuticals in Japan. The mesothelioma cancer cell lines which are NF2 deficient, were provided by a German group, while h-meso was provided by Joe Testa at FCCC in Philadelphia. Without those people, we wouldn't be able to develop those pak inhibitors and also unable to discover that pak is essential for the growth of NF2 and NF1 tumors. I think I had better stop talking here, and would be happy to answer any questions. Thank you very much for your kind attention!

Paul: We have 10 minutes for questions.

I skipped so many things. So I think many things may remain unclear. Even any technical or simple question will be welcome.

Rosemary: can I just suggest with the ic50 if you explain a little more about fk228 and how that is in comparison with something else. People are not clear to a point.

Regarding the ic50 of a drug called gleevec, which is good for a leukemia called cml (chronic myelogenic etc). The gleevec was developed by novartis in switzerland. Ic50 is about 50 nm. So compared with that of fk228 which is around 5 pm, gleevec I 10,000 times less potent than fk228(fk228 is 10,000 times potent as much as gleevec). Any other questions? Yes?

Cindy: first of all, I want to say thank you. You were very clear on your presentation. I wanted to clarify. You say pak 1 inhibitor, if it will help NF2, will it also help NF1, both diseases? You said it would help NF1 and NF2?

Yes. Fk228 helps both NF1 and NF2. And we are testing in mice to confirm that.

Marie: my understanding is that it takes a long time for a drug to get from clinical trials, particularly in the united states for them to approve it, and maybe some people would actually like to participate, be willing to participate in the clinical trial in the hope that it would help. How does one go about being able to participate in a clinical trial?

Well, I'm not a clinician, so I am afraid that I may not be able to give you the right answer. Probably Dr. Chan will give you a more appropriate answer. I presented you some of the chemical compounds, like fk228, or a virus which is already in clinical trials phase 2 or phase3. That is actually very close to the FDA's approval. With a few other compounds, we are doing animal experiments, and not clinical trials that probably takes more time, maybe 5 or 10 years for them to appear in market. Some chemicals are already in clinical trial phase 2 and phase 3, and probably become available for you, NF1 and NF2 people, before other chemicals become available. So that's the time scale. As I say, Dr. Chan can probably tell you more details.

Cindy: One more question. As a scientist, do you know how tnf can inhibit the cancer's growth? Do you understand what i'm saying?

She's asking whether tnf, tumor ecrosis factor, is useful for the treatment of NF1 and NF2.

I don't think it is effective from a point of basic scientist's view. The usefulness of the tnf is very limited. Ironically, the current director of our own institute is the person who initially developed that tnf. It's not working. That is why he's eventually given up.

Nf support groups, Karen can get you on the mailing lists and get you involved, answer any questions you have.

Dr. Hiroshi Maruta is the Head of the Tumor Suppressor Laboratory at the Ludwig Institute for Cancer Research (Melbourne branch). He is a molecular oncologist/biochemist who is an expert in the cell-signaling of tumors, and has written many books including editing the definitive text, "Tumor Suppressing Viruses, Genes and Drugs." Dr Maruta has been studying the tumor promoting role of the enzyme PAK for the last 20 years and in 2002, his group found that the NF2 protein, Merlin, is a natural inhibitor of PAK. Whilst continuing their drug development of new PAK inhibitors, his group has also been screening other drugs, in an effort to identify effective therapy sooner for the NF community.

This paper was originally presented at the ALDAcon held in Burlington, Vermont in September of 2004.