Reading through premature stop codons with PTC124. Project Catalyst to find more Duchenne drugs.

Interview with Ellen Welch PhD., Diane Goetz, and Neil Almstead PhD.

This interview was recorded at the company PTC Therapeutics in South Plainfield, New Jersey (about 20 miles west of New York) by me, **Guenter Scheuerbrandt** PhD., on 21 July 2008 after the annual meeting of PPMD, the American Parent Project Muscular Dystrophy, in Philadelphia. The following text is an edited and shortened version of the original interview. It has been approved by PTC for the information of patients, their families, and care-givers. My questions are written in italics, the answers of **Ellen Welch**, Associate Director, Genetic Disorders; **Diane Goetz**, Director, Patient and Professional Advocacy; and **Neil Almstead**, Senior Vice President, Chemistry, in normal print. The chapters about PTC124 and Project Catalyst, as they appeared in my last report "Research approaches for a therapy of Duchenne muscular dystrophy" are shown at the end of this text and should be read as an introduction to this interview.

This interview will be read mainly by the Duchenne patients and their families. We do not need to repeat what I explained in my last Duchenne research report about PTC124 and Project Catalyst but rather talk about the future, the further development of your work for finding therapies for Duchenne muscular dystrophy. It should give hope to the families that there will be effective treatments in the not too distant future.

The beginning of PTC Therapeutics. *My first question is, when was PTC started? Why did you select Duchenne muscular dystrophy and cystic fibrosis as the first targets of your research?*

We just had our 10-year anniversary in April. Our company, PTC Therapeutics, was founded by Dr. **Stuart Peltz** and Dr. **Allan Jacobson**, who based it on the technology they used in their own laboratories. They were studying the way RNA was making proteins in the body, and they were convinced that it would be possible to use substances, which had small molecules, to repair the mistakes in the messenger RNA that were causing inherited diseases.

The reason we got into cystic fibrosis was that we were collaborating with Dr. **David Bedwell** at the University of Alabama, who worked with a clinical group on cystic fibrosis. Most importantly, he had developed a laboratory mouse that contained human cystic fibrosis genes with a nonsense mutation, that had created a premature stop codon. And he realized that one could overcome this stop with the antibiotic gentamicin by reading through the premature stop, so that the production of the CFTR protein was restored, which is missing in patients with cystic fibrosis.

And how did we get into Duchenne? That was because professor **Lee Sweeney** at the University of Pennsylvania had shown with **Beth Barton** in 1999, just as the company started, that gentamicin could also read through the premature stop codon in exon 23 of the dystrophic mdx mice. These mice then could make again the full-length dystrophin protein, and their symptoms were reduced.

So we called Lee and asked him whether he would mind testing one of our compounds on his mouse. He said: "Oh sure, send the compound to us and we will test it for you." Lee is a wonderful person. He does not care whether he publishes a paper or not; the important thing for him is to develop drugs for patients.

The first targets: Duchenne muscular dystrophy and cystic fibrosis. *So you started to develop a small molecular drug for Duchenne and cystic fibrosis at the beginning of your work?*

And we still do. These two projects are going forward at the same pace. These two diseases are the most frequent genetic diseases in childhood. There is a tremendous need to find something here. And we were happy that we had animal models we could test to find out whether our compounds were actually working. So this is why we went forward with these two diseases.

About two to three years ago, we went to the FDA, the federal regulators, and explained to them that we had a small molecule that could read through a nonsense mutation, and we would like to go forward with muscular dystrophy and cystic fibrosis. They said that because the drug would be for long-term treatments during the lifetime of the patients, we should develop it separately for the two diseases, and that we demonstrate first that it is safe. So we designed clinical trials which are now underway for Duchenne and cystic fibrosis.

In Duchenne, about 13% of the patients could be treated with PTC124. What is the situation in cystic fibrosis?

In general, cystic fibrosis appears only in the white population, and about 10% of the patients have a nonsense mutation. But in Israel, the percentage is in the 60% range, because there is a founder effect there. The genetic situation of cystic fibrosis is different from muscular dystrophy. The gene for muscular dystrophy is X-linked, it is on the X chromosome. Boys have only that one dystrophin gene. In cystic fibrosis, CF, the CFTR gene is on chromosome 7, so everybody has two genes and the CF mutations in the two genes can be different. One mutation can be the very frequent delta-F508, leading to a deletion of the critical amino acid phenylalanine in the protein, and in the other gene, one can have a nonsense mutation. Even if there is only a premature stop codon in one gene and another mutation in the other, PTC124 will probably help.

How does PTC124 work? *Before we discuss Project Catalyst, I have one biochemical question: When PTC124 is reading through a stop codon, which appears when an amino acid codon has one of its three genetic letters changed by the mutation, this codon is not used anymore for protein synthesis. Doesn't the dystrophin or the cystic fibrosis protein CFTR then have just one amino acid less?*

Oh, no. When the ribosome, which makes a protein by translating a messenger RNA, comes to a premature termination code, it will stop and thus produce a shortened protein. PTC124, when it is present, does not cause the ribosome to skip the premature stop codon; it lets it put in another amino acid, which generally is well tolerated unless it changes a critical part of the protein. But, generally that is not a problem. The stop codon is not hopped over like an exon in exon skipping. Another amino acid is put in.

Is it known how PTC124 works on the molecular level?

The exact molecular mechanism is unknown, but we can guess and we generally understand what PTC124 does. The FDA does not require that we know the exact mechanism to move PTC124 forward.

You have probably tested whether PTC124 doesn't do anything else than just reading through premature stops. Are you really certain that it does not do something else on any other of our about 20,000 genes? And why is it not reading through normal stops?

We haven't looked at all the 20,000 other genes. What we were looking for was whether PTC124 could also read through a normal termination codon at the end of the messenger RNA. So we checked a number of genes to see whether PTC124 would sometimes make the protein longer, make an extension. But we found nothing, there were no extensions. Then, we administered PTC124 at extremely high doses to rats and dogs and checked different tissues – brain, heart, intestines – to see whether in the living animal PTC124 was reading through the normal termination codons. Again, we saw nothing. So we were confident that PTC124 was acting specifically enough only on premature stops.

Why is that so? The messenger RNA is not a straight structure. It is really a circle so that the beginning of the RNA interacts with its end. And when the ribosome gets to the normal termination codon, it is close to the beginning of the RNA. But a premature stop codon appears when a nonsense mutation appears in an amino acid codon in a region where the ribosome translates and makes the protein. So the two types of stop codons, the normal, and the premature one, are in a very different environment. And that is what makes PTC124 so specific, it is very, very selective.

Project Catalyst. *Please explain now what you are doing in Project Catalyst.*

Lee Sweeney was actually the one who suggested that PTC work with **Pat Furlong**, the president of PPMD, on Project Catalyst in order to identify other drugs against Duchenne, on targets that should be either up- or down-regulated.

As you explained in your report, we work now on five targets, but we used to work on six until recently. However, for one of them, phospholamban, which was to sustain cardiac function, we could not make any chemical changes for optimizing it. So we dropped it and are now trying to up-regulate its binding partner, SERCA2a, which would improve the contractile function of the heart muscle. As many patients have cardiac problems, some even quite early, we thought that if we are to make a drug mixture, a therapeutic cocktail, we would need something that has a positive cardiac effect. The other targets we are working on are to up-regulate the muscle-specific growth factor IGF1 and to down-regulate myostatin. Then, we want to make the muscle membrane stronger, so we hope to replace dystrophin by up-regulating utrophin. And more alpha-7 integrin would stabilize the membrane from the outside.

The targets you have chosen for Project Catalyst, they could be applied to 100% of the boys, and not like PTC124 to only 13% of them?

Yes, they will be for all the boys. And we could probably combine them with PTC124. Why not?

Automatic drug screening. *Now we come to the automatic screening. We should tell the families how this kind of science is really done. In films with scientists, they are normally shown boiling something or pouring some liquid from one test tube into another. Now it is so much different with lots of computer work and other electronic techniques. Can you say something about this without being too technical?*

Our approach to drug discovery, as in larger companies, is to look for a needle in a haystack. We have a "library", a collection of a tiny bit of about 200,000 substances. One can buy them from companies specializing in collecting or making them. We select from their catalogues those that have certain drug-like properties. Generally we want them as a powder; they should be water-soluble, and the size of their molecules should be less than 500 atomic mass units, that is, one of their molecules should weigh less than 500 hydrogen atoms. And there are other properties we are looking for.

We get these powders in vials but store them refrigerated in well plates. They are transparent rectangular plastic trays, about 6 times 10 cms, with 96 wells, indentations like tiny test tubes. The high-throughput automatic screening machine then transfers some of the contents of every four of these 96-well plates into larger plates with 384 wells, and use them for the screening procedure.

The wells are labelled with barcodes to identify their contents. The machine reads the barcodes and thus keeps track of the compounds through the testing procedure. This is important because after the test results are in, it must be possible to find those wells which contain the "hits", substances that showed the desired activities. We then can get more of the material of the hits for further chemical procedures.

Now, we will explain what kind of test procedure the screening machine uses for finding the hits, those substances which, after optimization, may later be able to modify the five targets we selected. As it is not practical to measure the biological activities of the targets themselves, for instance the up-regulation of utrophin or the down-regulation of myostatin, we are using a single biochemical marker, an indicator that can be used instead to determine the activity of all the five targets, and which also helped us to find PTC124. This marker is the enzyme luciferase, which normally produces the light of the fireflies. For our tests,

we combine the gene for the target with the gene for luciferase in such a way that the up- or down-regulation of the target molecules in isolated muscle cells by some of the thousands of tested compounds also causes a corresponding up- or down-regulation of the luciferase. It is much easier to measure the intensity of light produced by this firefly enzyme than to measure the results of a complicated biological reaction.

For actually doing the screening test, a person puts the muscle cells into the high-throughput screening machine. Then he stacks a whole series of the 384-well plates, which already contain the substances to be tested in their wells, at the entrance of the machine. The machine takes one plate after the other every few seconds, fills each well with some of the muscle cells, and then lets the substances to be tested react with the cells for a certain time at a certain temperature: it "incubates" them. At the end of the reaction, a quite large camera takes an image of the firefly light from all the wells of an entire plate and sends the readings of the light intensities to a computer for calculation the final results and recording them.

Optimizing the active compounds. *And now, how are you getting at the hits, and how are you processing them further?*

At the end of a test run, we have many plates that have some active compounds on them, the hits. To get them out, we use a so-called cherry picker, with which the machine picks up a small amount of the hit from its well and transfers it into the well of a new plate. This new plate will contain only hits, and we then repeat the primary screen on these hits to confirm that the activity is still there. If that is the case, we generate a concentration curve by testing different amounts of the hit substance that was used in the first test. If we see a nice dose response, that is, more activity with more substance, we are confident that this hit is already a bit drug-like from the beginning.

How many hits are you getting?

Usually we will have somewhere around 1,000 to 2,000 hit compounds picked out of 200,000 tested substances. And usually about 50% give reproducible dose responses.

And now, we are doing secondary assays. For this we use the real biological reaction of the target, not the luciferase test, to see whether such a reproducible hit is not only active in the screening test but is really regulating the activity of the target protein. Only 20 to 50% of the remaining compounds will be confirmed in this way. We just want to make sure that it is really doing what we want.

And they will then be optimized?

Yes, because now the chemists start to get involved. The first step is to determine whether or not these compounds really have properties that will allow them to be converted into drugs. We check their toxicity in cell culture, and we make certain that they can easily be synthesized. If they are natural products, they might be too complex. We check whether they are water soluble enough so that they are readily dissolved and absorbed once they get into the stomach. In general, we follow known guidelines for properties of potential drug compounds. The chemists then go back to our library and look for compounds that have a similar molecular structure as the active hits and test them again. They then start to re-synthesize the molecules, confirm their activity, and finally modify their structure, which may or may not enhance their activity. At the end of this work, which may take several years, we perhaps have gone from 100 compounds all the way down to 5 to 10 really promising ones for each of the targets. This is a very severe cliff to come down.

How many optimized or to-be-optimized potential drugs for each of the five Project Catalyst targets do you now have? And how long will it take until clinical trials with Duchenne patients can be started?

We currently have identified several molecules of interest for each target. We are in the process of testing these molecules in animal models of disease. We need to continue to optimize these molecules until we are satisfied with their activity and safety. There are many steps between now and clinical trials, so it is difficult to predict when we would enter clinical trials at this time, but it will be several years at least.

The international PTC124 trial. *Now let us come back to PTC124. The details of your long-term international trial are described in my last report (see the attachment to this interview). In short, 165 patients in three groups will be treated for 48 weeks with two doses of PTC124 and placebo. Has this trial been started?*

It is being started on 38 sites and about 20 are selecting patients now. We have a steering committee to oversee the trial and to evaluate the results. In Europe, Drs. **Kate Bushby** in Newcastle and **Thomas Voit** in Paris belong to it and in the US Drs. **Valerie Cwik** of the MDA and **Giovanna Spinella** of PPMD. Every patient will be treated for almost a year. After that, all the boys, also those who were on placebo, will be able to receive the drug during the extension of the study, probably the highest dose, 80 mg/kg/day in three daily portions of 20, 20, and 40 mg/kg/day.

The final results will probably be available in the winter or spring of 2010. We will then be able file an NDA, a new drug application, with the FDA roughly two years from today. Since we will have an accelerated approval process, we should anticipate, although there is no guarantee, that we will get approval within about six months afterwards. Of course, there are many things that can go wrong and prevent the approval.

So we could expect approval by the end of 2010?

Yes, at the end of 2010. And we will also submit to the EMEA, the European medical agency, for the European countries. For many other countries, we will have to get involved in their regulatory processes. But much of this will be done in collaboration with our new partner, Genzyme, which is a much larger company than PTC and which has operations in many different countries of the world.

Will PTC124 be expensive?

It would be premature to speculate on the pricing of PTC124 at this point. Right now we are concentrating on doing the trial. When we know that the drug works and is safe, then we will be able to talk about it.

What is PTC124? *One last question: What does PTC124 mean?*

PTC is the abbreviation of "post transcriptional control" and 124 comes from its chemical name. It is a 1,2,4-oxidiazole, consisting of a five-membered ring with two car-

bon, one oxygen, and two nitrogen atoms linked to two benzene rings carrying two small side chains. The structure is mentioned in the detailed 2007 *Nature* publication, and, obviously, it is patented.

Some final words. *I think it is now time for some encouraging words for the families at the end of this interview.*

Of course, we are excited about the progress we have made with PTC124. We are trying to move forward as quickly as we can to get it to all the Duchenne boys who need it. We also are excited about the prospects of Project Catalyst. For the boys who do not have a nonsense mutation, it is important to bring these other drugs forward for them, for 100% of them. Of course, we are working also as fast as we can to finish these potential drugs. Their development will be faster than for PTC124, because their clinical path is not very different, and we have more experience now. But it will also depend on the results of the present international PTC124 trial.

We have focused much of our effort on the identification and development of compounds that would treat different muscle diseases, specifically Duchenne muscular dystrophy, but spinal muscular atrophy and myotonic dystrophy as well, and will continue to work on cystic fibrosis. This distinguishes us from large pharmaceutical companies which focus much of their effort on lifestyle drugs. However, we at PTC do everything to find therapies for perhaps not very frequent diseases but really serious ones that are difficult to treat. That is, and remains, important for all of us.

When I am talking and writing to families with Duchenne muscular dystrophy, I realize what real problems are. If I compare that with my problems of daily life, I understand that problems I can solve, even if that is difficult, are nothing against the problems of having a boy with Duchenne dystrophy for whom one has to care for many years.

We think this is also the motivation for our researchers to work on a disease like that. That makes our work meaningful and possibly part of history.

Thank you very much, also on behalf of many, many families and of everybody who will read what you have explained to all of us

As an introduction to the interview, two chapters about PTC 124 and Project Catalyst are reproduced here, which are part of my last report "Research Approaches for a Therapy of Duchenne Muscular Dystrophy" published in May 2008. The entire report together with the three previous ones in English, German, and Spanish can be seen under www.duchenne-information.eu on the internet. In these reports, the basic biochemical and genetic facts of Duchenne muscular dystrophy are also explained. Those who wish to receive my future reports and interviews as soon as they are ready should send me their email address to gscheuerbrandt@t-online.de.

Reading through premature stop codons with PTC124.
In about 13 to 15% of all Duchenne patients, the disease is caused by a nonsense mutation in the dystrophin gene. This type of mutation is a single-point change that results in the introduction of a premature stop codon into the dystrophin mRNA. Such a premature stop codon causes the protein synthesis to shut down prematurely before the new dystrophin is fully assembled. The incomplete dystrophin is too short to fulfil its normal function, it is destroyed, and Duchenne muscular dystrophy develops.

PTC Therapeutics, Inc, a company in South Plainfield, New Jersey, has – under the direction of Dr. ***Langdon Miller*** – developed a drug, *PTC124*, which allows the protein-making system in the cell to *read through* such a premature stop codon in the mRNA, so that the full-length protein can be made. Such a treatment is different from gene therapy or exon skipping. To decide whether a boy with Duchenne muscular dystrophy can benefit from PTC124, the presence of a premature stop codon - TGA, TAG, or TAA - in one of the exons of his dystrophin gene must be proven by genetic analysis.

Details about this new drug, including its molecular structure, have been published in Nature in May 2007 with a commentary. PTC124 is a white crystalline powder which can be taken by mouth after mixing it with water, or milk. PTC124 was discovered using an automated screening program in which about 800,000 compounds of low molecular weight were tested for their read-through ability. One of the most effective among the active compounds, PTC124, was optimized chemically and then extensively tested in the laboratory. In pre-clinical experiments in muscle cultures, dystrophin was produced. In mdx mice, which have a premature stop codon in exon 23 of their dystrophin gene, it was shown that PTC124 induces full-length dystrophin production, resulting in reduced injury during muscle contraction, and decreases the creatine kinase (CK) activity in the blood. Thus, PTC124 may help the muscle cells to overcome one of the genetic causes of Duchenne muscular dystrophy.

PTC124 does not read through normal stop codons which have a different structural environment compared to premature stop codons. Toxicity studies in mice, rats, and dogs with high doses of the drug have shown an acceptable profile for continuing clinical development of the drug.

A phase-I clinical trial of PTC124 was performed in 61 healthy 18- to 30-year old adult volunteers who received the drug 3 times per day for 2 weeks. With this treatment, a serum concentration of 2 to 10 micrograms/ml could be maintained that was known to be active in mdx mice. Doses of up to 100 mg/kg/day were well tolerated by these healthy adults without serious side effects. This dose is larger than that planned to be given to Duchenne boys.

These results allowed the start of a phase-IIa clinical trial with Duchenne boys, which was performed between December 2005 and May 2007, and in which 38 boys, 5 to 17 years old, participated. They were a representative group of patients, 33 could still walk, 29 received steroids, 26 had the stop codon UGA, 6 UAG, and 6 UAA between the exons 6 to 70. The trial was not designed to produce any therapeutic benefit. Six boys received 16 mg/kg/day PTC, 20 boys 40 mg/kg/day, and 12 boys 80 mg/kg/day divided into 3 portions per day. The patients were clinically evaluated for up to 21 days before the treatment, then received the drug for 28 days, and finally had follow-up examinations again for 28 days. Muscle biopsies were performed before and after the treatment on the *extensor digi-*

torum brevis (EDB) foot muscle to check for the restoration of full-length dystrophin production. Before the treatment, muscle tissue from the first biopsies was treated with PTC124 in the laboratory. The expected dose-dependent increase of full-length dystrophin was detected in the tissues from all boys.

The expected dose-dependent increase of full-length dystrophin was detected in the muscle tissues from all boys when tested in the laboratory. The analyses of the muscle tissue from the biopsies after the treatment detected in 19 of the 38 boys, with qualitative increases of new dystrophin expressed at low levels. The main reasons why new dystrophin was not found in all boys and not in larger amounts could be that the treatment period was too short and that the EDB muscle was probably not the best one to be analyzed because it has such a low rate of degeneration and regeneration. However, all boys showed a reduction of the blood CK level during treatment. Blood CK increased again after the treatment as expected for a drug that has to be taken continuously. Some parents and teachers observed that 2 to 4 weeks after the rather short treatment, the boys showed greater activity, increased endurance, and less fatigue than before the treatment. While these anecdotal results must be considered cautiously, the time course of symptomatic changes suggested a drug effect. Some mild to moderate adverse effects were observed but these were not clearly caused by PTC124 and were not clinically relevant.

To understand the long-term risk and benefits of PTC 124, a randomized controlled long-term phase-IIb clinical trial is now being started. This trial will enrol 165 patients who are at least 5 years old and still ambulatory (able to walk ≥75 meters). Boys who are on corticosteroids will be allowed to continue that treatment. Participants will be randomised to one of 3 study groups: higher-dose PTC124, lower-dose PTC124, or placebo. Treatment will continue for 48 weeks. The primary outcome measurement will be the distance the boys can walk in 6 minutes, comparing the results before the treatment with the same measurement during the treatment. There will be 10 additional secondary outcome measurements. After completion of the trial, all patients, including those who were on placebo, will receive long-term therapy with the higher dose of PTC124.

For this trial, an international steering committee has been established which will organize and oversee the collaboration of many clinical centers in Europe, Australia, Israel, Canada and in the United States. Drs. *Kate Bushby* and *Thomas Voit* are the European experts in Duchenne muscular dystrophy who are participating in the steering committee. The trial is ongoing in the US and will soon be opened in other countries. If this large phase-IIb trial shows good therapeutic effects, marketing approval will be sought from the regulatory agencies FDA in the United States and EMEA in Europe.

Welch EM, Barton ER, Zhuo J. PTC124 targets genetic disorders caused by nonsense mutations. Nature 2007; 447;87-91.

Schmitz A, Famulok M. Ignore the nonsense. Nature 2007;447;42-3.

Project Catalyst is a program of PTC Therapeutics for finding and developing small chemical compounds as drugs for a therapy of Duchenne muscular dystrophy.

Project Catalyst, directed by Dr. ***Ellen Welch***, was started in May 2004 to identify with automatic screening methods among several hundred thousand compounds those that could up- or downregulate the production, the *expression*, of four biological targets in muscle cells and thus maintain and improve muscle structure and function in Duchenne patients. The down-regulation of *myostatin* and the up-regulation of the muscle specific *insulin-like growth factor* IGF-1 would promote muscle growth and regeneration. The up-regulation of *utrophin* and *alpha7-integrin* would stabilize the muscle membrane and thus improve muscle function.

The automatic screening methods for finding these potential Duchenne drugs use a newly developed test procedure which measures the light intensity of a reporter-protein, the enzyme luciferase from fireflies. A small number of compounds with at least some of the desired properties have now been optimized in the laboratory. In addition, work was initiated on another protein target, the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) to help maintain proper contractile function of the heart. These very promising potential drugs will be further optimized so that phase-I clinical studies with Duchenne boys could start in the near future.

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