Dr. Trent D. Stephens Ph.D. and Dr. Bradley J. Fillmore had an article published in Teratology 61 : 189-195 (2000) entitled "**Hypothesis: Thalidomide Embryopathy - Proposed Mechanism of Action**". Simply put, the published article proposes how thalidomide affected the foetus when taken by a pregnant woman.

Dr. Trent D. Stephens Ph. D. agreed to offer an article written specifically for the TVAC ACTION Newsletter to describe his findings.

Dr. Trent D. Stephens Ph. D. agreThe Thalidomide Victims Association of Canada and this publication thank Dr. Stephens for his cooperation in this matter.ed to offer an article written specifically for the TVAC ACTION Newsletter to describe his findings.

RE: THALIDOMIDE MECHANISM

by Dr. Trent D. Stephens Ph. D.

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Written by : Glenn Alford, March 3, 2000

October 1st, 1957, three days before the Russians launched Sputnik into the October sky, changing our lives forever, Chemie Grünenthal formally launched their new sleeping pill, Contergan, onto the world market. Although few people were aware of this second launch, and no one knew its implications, it would also permanently change our lives and perspectives. I was nine years old when these two launches occurred.

In August 1962, Dr. Helen Taussig, of Johns Hopkins School of Medicine, published a paper entitled "The Thalidomide Syndrome", in *Scientific American*. I was a freshman at Raft River High School in the tiny farming community of Malta, Idaho in the fall of 1962. The rack of current journals occupied one very small corner of our small high school library/study hall. About the only scientific journal my high school subscribed to was *Scientific American*, but I read it religiously. I was profoundly impressed by the drawing of a "thalidomider", in Dr. Taussig's paper.

Ten years later, as an undergraduate at Brigham Young University, I read a riveting paper by Dr. Edgar Zwilling published in Developmental Biology, on the advantages of studying chick limb development as a means of understanding biological form. From that time on, normal and abnormal development of the limbs became my passion, my obsession; and, of course, solving the mystery of thalidomide's mechanism of action was part of that obsession. I eventually went to work on my PhD under the direction of Dr. James W. (Jay) Lash in the Department of Anatomy at the University of Pennsylvania because he had published several papers on the mechanism of action of thalidomide. But Jay had reached an impasse in his research and could not identify any way around it. As it would turn out, the riddle couldn't

possibly have been solved at that time. The basic molecular information wouldn't become available for more than twenty years. While I was at Penn, the thalidomide issue was placed on a back burner and I worked on another question : the origin of the limb. That's another story.

In the meantime, interest in thalidomide was waning world-wide and the number of papers being published about the drug declined from about one hundred papers in 1967 to less than twenty five in 1980. In concluding her 1962 Scientific American paper, Dr. Taussig stated, "For most people the story of thalidomide has ended". Of course, the story was far from over for thalidomiders and for the few of us whose passion it was to solve the mystery of how the drug worked.

In 1986 the Teratology Society sponsored a symposium to commemorate the 25th anniversary of the thalidomide disaster. Some of the key players, such as Dr. Widukind Lenz and Dr. Frances Kelsey participated in the symposium. It was an excellent symposium, and it was a thrill to meet the key players in the history of the drug; but in the final analysis I felt that something should have been said about thalidomide's mechanism of action. I made such a comment to Dr. Lewis Holmes, president of the society, and Dr. Robert Brent, editor of the journal. They invited me to submit a paper on thalidomide's mechanism to be published with the symposium proceedings. I had not actually intended to write such a review myself, but then I concluded, why not. I had already published about fifteen papers dealing with various issues surrounding thalidomide's possible mechanism. So I wrote the review. I surveyed the literature indexed over a twenty five year period in Index Medicus, and identified 24 proposed mechanisms which had been advanced since the thalidomide incident. I had concluded that even though a number of hypotheses had been advanced, we really had no idea how thalidomide caused its embryopathic damage. The paper was published in Teratology in 1988 as part of the symposium proceedings.

Because of that review paper, I was invited to attend the 25th anniversary meeting of the European Teratology Society, held in Cannes, France, September 1997, and participate in their Thalidomide Symposium. My task was to update the drug's proposed mechanism of action since my 1988 paper. I reviewed the status of the original 24 proposed mechanisms and then discussed several more recent proposals, including some data from research in my own laboratory. Those newer studies were shedding much light onto the mystery. I concluded that although we still didn't have the complete picture, several critical pieces of the puzzle had recently been discovered and it wouldn't be long before they could be assembled to reveal the solution to the 40-year-old mystery.

It took twenty five hours for me to travel home from the meetings. During the entire twenty five hours I didn't sleep. My presentation at Cannes kept running through my mind. "The important pieces are all there," I reasoned, "All that remains is to find the

link that holds them together." "The final solution must be close, within reach, if only I can find it". I pulled out a yellow legal pad, and for most of the flight home I went over and over the list of puzzle pieces and their interrelationships.

Each time I went back over the known pieces of the puzzle, four pieces seemed to keep popping up as critical to the overall solution:

1. Thalidomide intercalates into DNA (the thalidomide molecule, which has a flat structure, can slide into gaps between the subunits of the DNA molecule, much like sliding a CD into the slot of a stereo system).
2. Thalidomide does not intercalate at random into the DNA but intercalates at guanine sites. Each DNA molecule is composed of thousands of subunits called nucleotides, of which there are four basic types: guanine, adenine, cytosine and thymine (DNA is much like a recipe written with a four-letter alphabet). Thalidomide, which is composed of three attached rings, two of which look in overall structure almost identical to guanine and adenine, does not attach in solution to cytosine and thymine and attaches more strongly to guanine than adenine.
3. Thalidomide has been shown to interfere with the production of certain proteins (as discussed below).
4. Thalidomide can interfere with angiogenesis, the formation of new blood vessels. Inhibiting the growth of new vessels in a growing limb bud would deprive the proliferating cells of critical nutrients, leading to a decrease in proliferation and a subsequent reduction in overall limb size. Even though these four puzzle pieces had been identified, it was not clear how they went together.

Data from several laboratories indicate that angiogenesis is dependent on the presence of paired cell-surface adhesion molecules called integrins, which look like a pair of submicroscopic lollipops protruding from the cell surface. Integrins, like all proteins, must be produced from a DNA template by the process of transcription of the DNA into RNA and the translation of RNA into protein. The process of making a protein from a DNA template is somewhat like transcribing a cake recipe from a book (DNA) onto a file card (RNA), followed by translation of the recipe (now contained in the RNA sequence) into a cake (protein). Data from Dr. Diether Neubert's lab, in Berlin, indicate that thalidomide can down-regulate the synthesis of at least some of the integrin subunits, at least one of which, called beta 3, is a critical player in angiogenesis. If thalidomide intercalates into guanine residues of DNA and interferes with integrin beta 3 synthesis, where would such intercalation have its greatest impact?

Transcription of a given stretch of DNA (which may be a gene, part of a gene called an exon, or more than one gene in tandem) is initiated by a protein called RNA polymerase II. This polymerase attaches to the initiation site (a specific set of three

nucleotides: TAC) for a given stretch of DNA and initiates the process of transcription. Polymerases are complex proteins composed of several subunits. In addition, other proteins, called transcription factors, or promoters, bind to a segment of the DNA upstream from the initiation site and help the polymerase find the initiation site.

Promoter proteins also attach to specific nucleotide sequences. The most common sequences are TATA or CCAAT, and approximately 91 % of all gene promoter regions contain one or both of these sequences. The remaining 9 % of gene promoter regions do not contain TATA or CCAAT sequences but contain/one, or often several of the sequence GGGCGG (called the GC box). Because thalidomide can intercalate into DNA most specifically at guanine-rich sites, the drug should intercalate into promoter regions with GC boxes far more often than into those with TATA and/or CCAAT boxes. Therefore, proteins, such as the integrin subunit beta 3 , which have been shown to be down-regulated by thalidomide, should fall into the 9 % minority group with GC-box promoters rather than the more common promoters with TATA and/or CCAAT boxes. As predicted, the promoter for the beta 3 gene is TATA-less and CCAAT-less, but rather contains multiple GGGCGG sequences. Intercalation of thalidomide into this G-rich promoter region of beta 3 gene could be enough to decrease transcription and subsequent translation of this gene into beta 3 proteins. Without this critical cell-surface adhesion protein, angiogenesis could be inhibited and truncation of the limb could result.

As already stated, integrins exist on the cell surface in pairs called heterodimers, which consist of one alpha subunit and one beta subunit. The integrin most directly involved in angiogenesis is called alpha v beta 3. NeubertAEs lab reported a down-regulation of beta 3 in embryonic primate (marmoset) limb buds exposed to thalidomide. However, they did not examine the other subunit, alpha v. Although it has been shown that down-regulation of beta 3 alone is sufficient to decrease heterodimer formation and inhibit angiogenesis, we decided to look at the alpha v gene promoteras well. As with beta 3, the alpha v gene promoter is TATA-less, CCAAT-less, and has multiple GC boxes. Therefore, it appears that the synthesis of both subunits of the heterodimer integrin cell surface adhesion protein are sensitive to inhibition by thalidomide intercalation into DNA. The system is susceptible to a double hit! The specificity previously missing from models of thalidomide's mechanism of action was beginning to emerge

It has been shown in several laboratories that both insulin-like growth factor type I (IGF-I) and fibroblast growth factor type 2 (FGF-2) can stimulate the production of the alpha v beta 3 integrin. It turns out that the gene promoters for both of those proteins are TATA-less, CCAAT-less, and rely on GGGCGG sequences for initiation. Therefore, thalidomide can not only directly inhibit integrin production, but can indirectly inhibit it by inhibiting production of the proteins that stimulate alpha v beta 3

production. However, other FGF's, such as FGF-1 and FGF-4, which are involved in limb development, but not in angiogenesis, have standard TATA and CCAAT boxes in their promoters, and therefore are not sensitive to thalidomide inhibition. The story goes on, and it gets even better.

In order for IGF-I and FGF-2 to stimulate integrin production within a cell, they must bind to cell surface receptors. The genes for the IGF-I receptor and at least one of the four FGF-2 receptors have TATA-less, CCAAT-less promoters, and have multiple GC-boxes. This is now becoming a familiar theme, but it doesn't end there. In order for IGF-I to bind to its receptor, it must first be attached to a protein called IGF-I binding protein (IGF-IBP). You guessed it, the IGF-IBP gene promoter is also TATA-less, CCAAT-less, and GC-rich. Furthermore, the IGF-I receptor stimulates the activity of an intracellular protein called IGF-I receptor substrate 1 (IRS-1). The IRS-1 gene promoter is also TATA-less, CCAAT-less, and GC-rich. As a result of our search, we have discovered that at least eight of the players in the sequence of events from growth factor stimulation to integrin cell surface adhesion proteins, which stimulate angiogenesis, are produced by genes that belong to the minority group of promoters that rely on GC boxes for gene function. Each step in this chain of events is potentially sensitive to thalidomide intercalation. The probability of linking eight steps in one chain of events all of which belong to this rare class of gene promoters is 4 x 10-9. Furthermore, with such a linked pathway, thalidomide does not have to affect any one part of the pathway to any great extent to have profound effects on the overall outcome. For example, if thalidomide decreased the efficiency of each step in the pathway by only 10 % the entire pathway efficiency would be reduced by more than 50 %. A 20 % reduction at each step would reduce the whole system by 80 %.

The model of thalidomide embryopathy we have presented unifies nearly all the previous models, provides biological relevancy, and provides biochemical and molecular specificity. This model can also explain the species specificity of thalidomide. For example, species may differ in the number of genes in the described pathway with GC promoters rather than TATA and/or CCAAT promoters. Rats and mice may not have as many genes in this pathway that have GC promoters, and therefore are less sensitive to thalidomide than are rabbits, humans, and other primates. In addition, it is well known that the formation of new blood vessels can occur via one of several pathways, only one of which was discussed in this paper. Other pathways do not depend on alpha v beta 3, and some have been demonstrated to be thalidomide resistant. It is known that there are distinct tissue differences in the choice of angiogenesis pathway. These differences may account for thalidomide's tissue specificity, affecting the limbs, eyes, and ears, for example, but having little or no affect on the developing lungs or brain. The choice of different angiogenesis pathways may also be species specific. The more classic view of thalidomide's species specificity is that species differ in the way they metabolize

thalidomide. Although this issue has not been addressed in this paper, it may be likely that metabolic products of thalidomide are either more able to reach the DNA or are better at intercalating into the DNA molecule than the native thalidomide. In addition, new data from Peter G. Wells'lab at the University of Toronto suggest that thalidomide embryopathy involves oxidative DNA damage, which appears to be species specific. Intercalation of thalidomide into DNA could be enhanced by DNA oxidation, especially at the eight position of the guanine residues, which extend toward the major groove where thalidomide intercalates. At present, insufficient data exist concerning species differences relative to a number of these proposals, and this should be an area of future research.