

The Effect of Thalidomide, an Angiogenesis Inhibitor, on the Estrus Cycle and Reproductive Function of Female Mice

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ABSTRACT

Thalidomide is an angiogenesis inhibiting drug that is being investigated as a possible treatment for various cancers and diseases. The effect that thalidomide could have on normal processes in the body that involve angiogenesis, such as the reproductive cycle of females, is not fully known. The purpose of this study was to observe and determine the effect of thalidomide on the reproductive system of female mice. Thalidomide was administered at 0 mg/kg, 60 mg/kg, 180 mg/kg, and 560 mg/kg for 20 days. During this time, the duration of the estrus cycle and the length of time spent in each of the four stages of the estrus cycle were assessed. Subsequent to euthanization, the ovaries and uterus were extracted, processed with zinc fixative, and embedded in paraffin wax. Immunohistochemistry, using monoclonal antibodies that target platelet endothelial cell adhesion molecule (PECAM-1), was performed to visualize the endothelial cells of the blood vessels. The dimensions of the organs were analyzed and the blood vessel density was compared using ImagePro and Adobe Photoshop software. Using SPSS 10.1 and one-way ANOVAs, no significant differences between the separate treatment groups were found for estrus cycle characteristics, organ dimensions, or blood vessel density.

INTRODUCTION

Cancer is a disease that is characterized by uncertainty. It can afflict any person, often regardless of age, weight, health or genetics. Cancer seems to sneak in on a person, doing much damage before it is even detected in the body. The uncertainty surrounding cancer causes it to be extremely frightening and very devastating to the people it affects. In the past century, cancer research has thrived and will help us continue to add to our mounting knowledge of this elusive disease. Discovering more effective treatments encompasses one part of the cancer research arena. Currently, a popular treatment concept being researched is that which was first generated by Judah Folkman in the early 1970s. In 1971, Folkman published a study in which he found that tumors are dependent upon angiogenesis to grow. Without the spread of new blood vessels into and around a tumor, it could only grow to approximately 1-2 mm in diameter (Folkman, 1971). Folkman followed these findings with a novel idea for cancer therapy – if angiogenesis could be prevented, the growth of tumors could be inhibited. Folkman proposed that treatment with anti-angiogenic drugs would introduce a new concept into cancer therapy. Instead of eradicating the cancerous tissue, the cancer cells would be starved of blood vessels and the tumor would be forced to remain at a small, harmless size. Cancer could evolve from being a disease that is cured to a disease that is managed (Folkman, 1972).

Since Folkman's initial studies, over 60 angiogenesis inhibitors have been found and are currently undergoing research (Marx, 2003). While anti-angiogenic drugs are

experiencing success in both laboratory experiments and clinical trials, there are still some problems that must be worked out before these drugs are fully accepted for cancer treatment. One of these problems involves the possibility of unknown effects of these drugs on the natural processes of the body that involve angiogenesis, such as wound healing and the reproductive cycle of females. Some of the factors of the body that are being targeted by anti-angiogenic drugs to prevent blood vessel formation are the same factors that are responsible for human health and normal functioning. It therefore seems possible that an angiogenic inhibitor would have some effect on the reproductive cycle of females.

The goal of my research was to evaluate the consequences thalidomide may have on the estrus cycle and reproductive function of female mice and the mechanisms through which thalidomide has these effects. Thalidomide was first synthesized in the 1950s by a German pharmaceutical company and was marketed as a sedative. However, pregnant women began using thalidomide as a morning sickness drug, as it helped to relieve their nausea. It was soon discovered that thalidomide is highly teratogenic, causing severe malformations in the children of those women who took the drug while pregnant. As a result, the drug never gained approval in the United States and was taken off the world market in 1961. Although much research has gone into determining exactly how thalidomide causes birth defects, there is not yet a clear answer. In recent years, interest in thalidomide has been renewed as it is showing promise for the treatment of leprosy. In 1998, the FDA approved thalidomide in the United States for the treatment of erythema nodosum leprosum (ENL). Currently, thalidomide is being studied as an angiogenic inhibitor and a possible treatment for many cancers, including multiple myeloma (Rajkumar et al., 2000), Kaposi's sarcoma (Little et al., 2000), renal cancer (Stebbing et al., 2001), Crohn's disease (Ginsburg et al., 2001), and more recently, prostate cancer (Macpherson et al., 2003). Although there have been multiple general toxicology studies on thalidomide (Teo et al., 1999; Teo et al., 2000), there is a lack of research on the possible effects of this drug specifically on the natural angiogenic processes of the body.

In 1968, a study was done on the influence of thalidomide on fertility and reproductive organs in rats (Samojlik, 1968). This research found that after just 15 days of treatment, thalidomide caused infertility in the rats and produced a lack of estrus or prolonged proestrus. Samojlik found that the uteri and ovaries of the treated rats were smaller than those of the untreated, and fewer corpora lutea were seen through histological examination. Samojlik's research was preliminary, however, and was never published in a scientific journal. Thus, my goal was to repeat Samojlik's research with mice in an expanded study to determine if similar effects would be observed.

In 2003, it was reported that thalidomide can cause amenorrhea in the female patient (Dharia et al., 2003). The disruption of reproductive cyclicity could have serious consequences for the female patient taking thalidomide, besides just making her infertile. The ovaries produce the hormones estrogen and progesterone, which have a direct effect on many other systems in the body besides just the reproductive system. These hormones are produced at varying levels depending on the stage of the reproductive cycle. In the mature female, estrogen is responsible for the proliferation of the endometrial lining of the uterus. This prepares the uterus for implantation of a fertilized ovum, if fertilization occurs. However, estrogen can have other effects throughout the body. Estrogen causes increased osteoblastic activity, which helps to increase the bone matrix and strengthen the

bone through deposition of calcium and phosphate. A lack of estrogen in older, post-menopausal women can lead to osteoporosis. Estrogen affects protein deposition, fat deposition and metabolic rate. It causes a slight increase in total body protein and an increase in the quantity of fat that is stored in the body. Estrogen also slightly increases the metabolic rate of the female. Beyond these functions of estrogen, recent research has shown that many important organs of the body display receptors for estrogen, including the reproductive organs, the bones, the liver, the brain, and cardiovascular tissue (Mendelsohn, 1999). This implies that estrogen has some sort of regulatory role involving these organs and tissues.

The goal of this research was to determine how thalidomide affects the reproductive cycle of female mice, which served as a model for female humans. My hypothesis was that thalidomide would disrupt the regularity of the cycle by inhibiting angiogenesis. The inhibition of angiogenesis would cause a decrease in the vascularization of the uterus and ovaries and would impair the functioning of these organs. Through its inhibition of angiogenesis, thalidomide would cause a disruption in the normal functioning of the female reproductive system.

MATERIALS AND METHODS

This research included several different elements - (1) monitoring of estrus cycle stages and duration during treatment with thalidomide, (2) assessment of ovarian and uterine weight, (3) histology of the ovaries and the uterus, (4) estimate of blood vessel density in ovaries and uterus and (5) analysis of dimensions of ovaries and uterus. Forty female mice were used in this study, with 10 in each of three experimental groups and 10 in a control group. The three experimental groups received 60 mg/kg, 180 mg/kg, or 560 mg/kg of (+/-)-Thalidomide (Sigma-Aldrich) in a honey paste at approximately the same time each afternoon. The control group received the honey alone. The mice were treated for 20 days and subsequently sacrificed via CO₂ asphyxiation while under isoflurane anesthesia.

Monitoring of Estrous Cycle Stages and Duration

Forty female mice were used, with all mice checked for regular estrus cycles before treatment began. The mice were allowed to acclimate for two weeks, during which the regularity of the estrus cycles was confirmed. The stages of the estrus cycle were determined by the inspection of vaginal smears (Allen, 1922). There are three major types of vaginal cells – leukocytes, nucleated epithelial cells, and cornified cells. Depending on what stage of estrus the female mouse is in, the vaginal smear will contain different types and amounts of these three cell types. In diestrus, the smear contains a stringy mucous with entangled leukocytes and a few nucleated epithelial cells. In proestrus, the smear contains small, round, nucleated epithelial cells either singly or in sheets, and few to no leukocytes. In estrus, the smear contains many large, cornified cells with degenerate nuclei. Toward the end of estrus, the smear contains masses of adherent cornified cells. Finally, in metestrus, the smear contains many leukocytes and a few cornified cells. Vaginal smears were taken daily at approximately the same time in the morning. The lavage method was utilized, where a small-tipped transfer pipet was used to insert a drop of distilled water into the vagina, and then the water was immediately

sucked back out, bringing with it some vaginal cells. Methylene blue was used to visualize the cells and the smears were analyzed using a compound microscope. In order to compare the estrus cycle characteristics of the different treatment groups, the treatment period of twenty days was split into halves and the number of days spent in estrus during the second half was subtracted from the number of days spent in estrus during the first half. The mice were weighed every week to ensure that the thalidomide was not causing weight gain or loss. Food and water were supplied ad libitum.

Ovarian and Uterine Weight

After 20 days of treatment, the mice were sacrificed via CO₂ asphyxiation while under isoflurane anesthesia. A cardiac puncture was performed on each mouse and centrifuged to obtain plasma. The plasma was then stored at -70°C for possible future analyses. The ovaries and uterus were dissected from each mouse. Attached fat tissue was removed to the best of our abilities before weighing the ovaries and uterus together. The uterus was then cut down the middle where the uterine horns connect. One half of the uterus with ovary attached was placed into a 10% Neutral Buffered Formalin fixative, while the other half of the uterus with ovary attached was placed into a Zinc fixative (Pharmingen).

Histology of Ovaries and Uterus

Half of the organs were fixed in 10% neutral buffered formalin while the other half were fixed in a zinc fixative (Pharmingen) for several days. The organs were then put through a distilled water wash and a graded series of ethanol for dehydration (protocol provide by S. Sessions, Hartwick College). Subsequently, the organs were cleared in xylene and embedded in paraffin wax. Using a microtome, the ovaries and uterus fixed with the zinc fixative were sliced at 15 um and placed on subbed slides. The organs fixed in 10% neutral buffered formalin were saved for possible future use.

Immunohistochemistry

A rat anti-mouse monoclonal antibody for platelet endothelial cell adhesion molecule 1 (PECAM-1) (Pharmingen) was used to visualize blood vessels. The antibody-protein interaction was visualized using a secondary biotinylated anti-rat Ig antibody, streptavidin with horseradish peroxidase, and DAB substrate (Pharmingen) (Figure 1). Subsequent to antibody staining, the tissues were stained with hematoxylin.

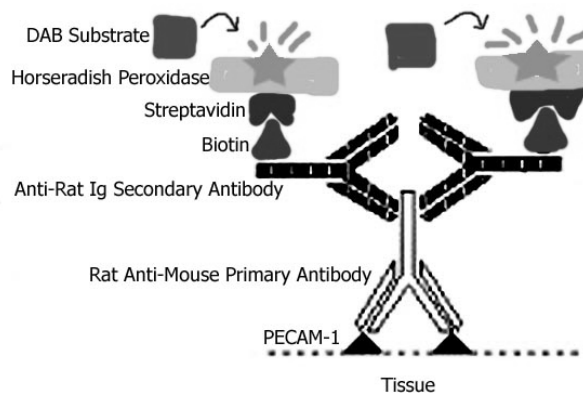


Figure 1. Diagram of immunohistochemistry.

Image Acquisition

Images of the stained slides were obtained through Spot v4.0 by Diagnostic Instruments, Inc. using a digital camera attached to an Olympus BX51 light microscope. Single pictures were first taken at a magnification of 40x to include the whole organ. These pictures were used for organ dimensional analyses. Multiple overlapping pictures of each organ were taken at a magnification of 100x. These pictures were used for blood vessel density analyses.

Organ Dimensional Analyses

In order to determine the dimensions of the ovaries and uterus, a computer imaging program called ImagePro was used. An ocular micrometer was first used to calibrate the software, and then ImagePro was used to determine the area of the ovaries and the uterus. Area was crudely determined by multiplying lengths of each organ (Figure 2).

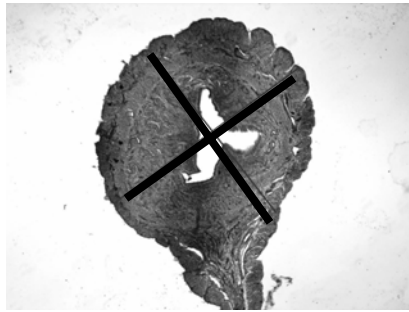


Figure 2. Organ areas were determined by multiplying lengths of each organ.

Blood Vessel Density Analyses

To determine the blood vessel density of the uteri, ImagePro was used to specifically pick out those pixels that were stained brown via the immunohistochemistry staining, and then color these pixels bright red. Adobe Photoshop was then used to determine how many pixels out of a total of 1.92 million for each picture were red. The percentage of red pixels, and thus the percentage of endothelial cells, were determined for each picture. Values from multiple pictures for a single organ were averaged.

Data Analysis and Statistics

Data were analyzed using SPSS 10.1. For each statistical test, a one-way analysis of variance was used to compare means from each treatment group. To determine where the statistical differences were located, a Tukey's post hoc test was used. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

In terms of the estrus cycle characteristics between different treatment groups, no significant differences were found. For all of the mice, the estrus cycle proved to be somewhat less predictable and more irregular. For the one-way ANOVA, the p value was 0.085 (refer to Figure 3).

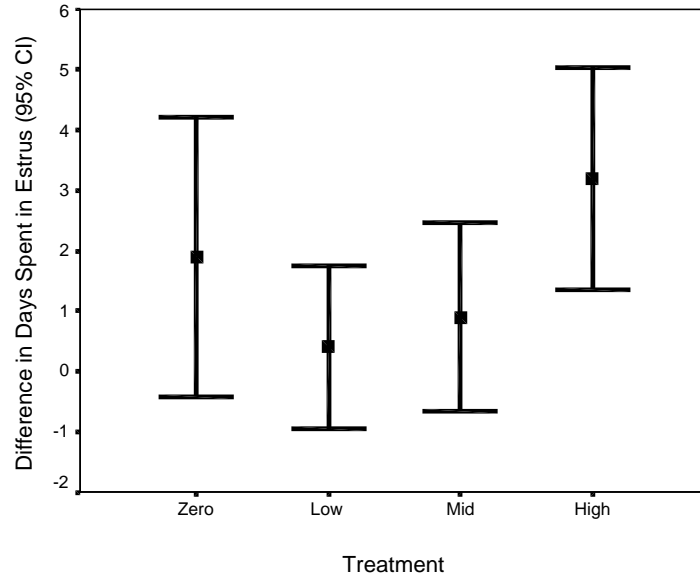


Figure 3. Difference in days spent in estrus between the first half of treatment and the second half for the four treatment groups are represented as means with error bars. (Zero = 0 mg/kg thalidomide; Low = 60 mg/kg; Mid = 180 mg/kg; High = 560 mg/kg)

When comparing the size of the ovaries and uterus between different treatment groups, no significant differences were found. For the ovaries, the one-way ANOVA gave a p value of 0.607 (refer to Figure 4). For the uteri, the one-way ANOVA gave a p value of 0.318 (refer to Figure 5).

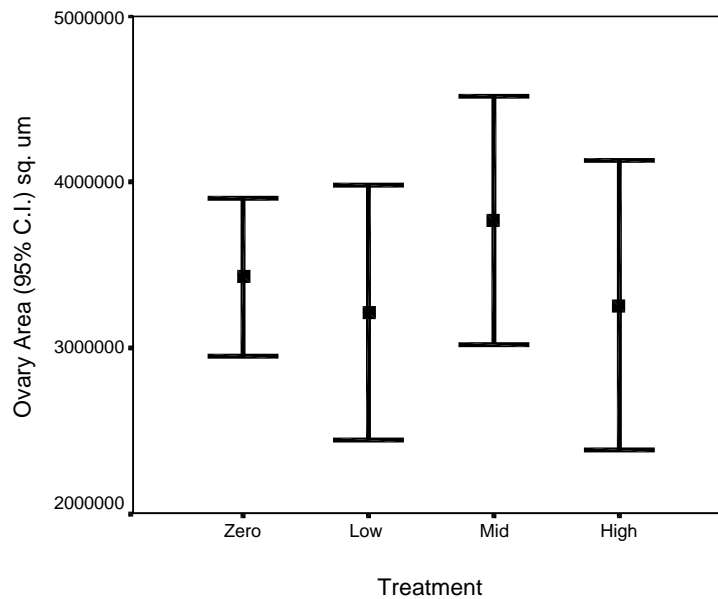


Figure 4. Differences in ovary area in square micrometers for the four treatment groups are represented as means with error bars. (Zero = 0 mg/kg thalidomide; Low = 60 mg/kg; Mid = 180 mg/kg; High = 560 mg/kg)

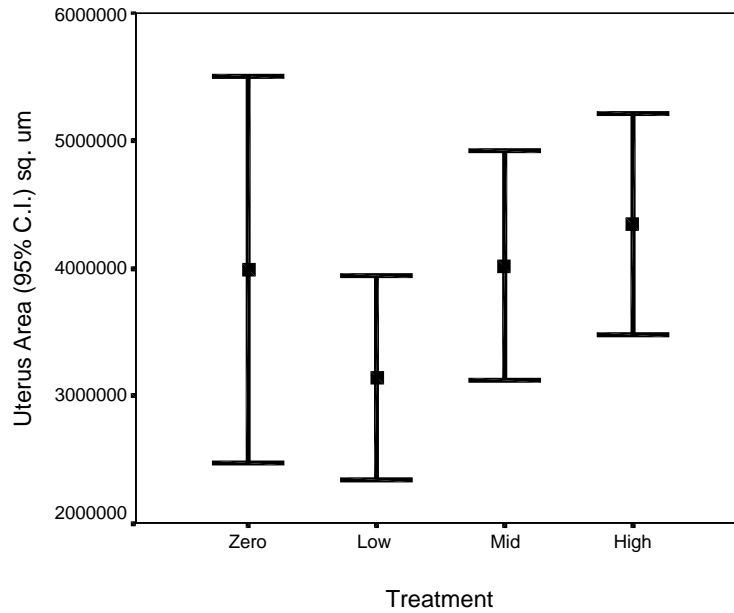


Figure 5. Differences in the uterus area in square micrometers for the four treatment groups are represented as means with error bars. (Zero = 0 mg/kg thalidomide; Low = 60 mg/kg; Mid = 180 mg/kg; High = 560 mg/kg)

To compare blood vessel density among the uteri, the number of pixels stained brown and therefore representing endothelial cells were compared among the different treatment groups. There were no significant differences found. The one-way ANOVA gave a p value of 0.591 (refer to Figure 6). The blood vessel density for the ovaries could not be compared because of the difficulty in staining the ovaries for endothelial cells.

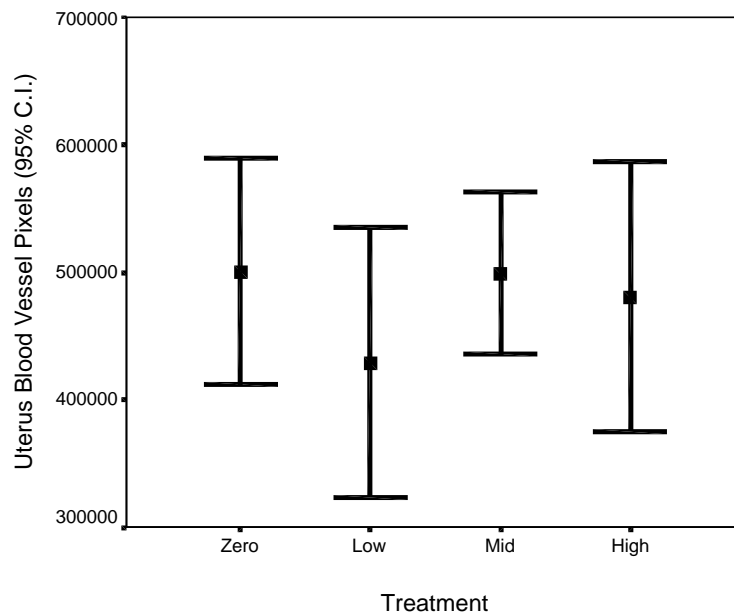


Figure 6. Differences in pixels representing endothelial cells in uteri for the four treatment groups are represented as means with error bars. (Zero = 0 mg/kg thalidomide; Low = 60 mg/kg; Mid = 180 mg/kg; High = 560 mg/kg)

The weights of the ovaries and uterus could not be analyzed because there was simply too much variation depending on how much fat tissue was attached to the organs. Although we attempted to remove the fat tissue to the best of our abilities, we found out afterwards that there were differences in weights based on which person had done the dissections. Therefore, we decided we could not use any results from the organ weights to assess the effects of thalidomide on the female reproductive system.

DISCUSSION

Interestingly, no significant differences were found between the different treatment groups, indicating that thalidomide did not have the effects expected. It is possible that the drug dosage was not high enough or was not administered for a long enough period of time to see the changes we were looking for. It's also possible that thalidomide affects the reproductive function of females in ways other than altering the vascularity of the organs. Perhaps there's an alteration in hormone production by the ovaries, and this in turn disrupts the reproductive cycle. Through correspondence with Dr. Steve Teo from Celgene Corp., the company that produces thalidomide, it was discovered that thalidomide seems to be very non-toxic to mice. Dr. Teo was able to administer up to 3,000 mg of thalidomide per kilogram of mouse per day for up to thirteen weeks without seeing any obvious toxicity in the mice. Therefore, it is also possible that mice metabolize thalidomide in such a way that it doesn't have the effects in mice that it has in humans. In future related research experiments, I would recommend either using a higher dosage, a longer treatment time, or a different species. I would also recommend looking at concentrations of estrogen and progesterone in the plasma of the treated individuals to get an idea of how thalidomide might affect ovarian production of female sex hormones. Because there is evidence from the case study by Dharia et al. (2003) that thalidomide can cause amenorrhea in human females, it is important that we continue to study this topic to understand just how thalidomide has these effects.

A second mechanism whereby thalidomide may have its effects should be presented here, although it was not analyzed specifically in this study. In 1991, it was discovered that thalidomide inhibits the production of the protein tumor necrosis factor alpha (TNF- α) in human white blood cells (Sampaio et al., 1991). TNF- α has been found to play a major role in the reproductive cycle of females (Okuda et al., 2003). Okuda found that the corpus luteum, a transient ovarian organ, contains many receptor sites for TNF- α , indicating that TNF- α plays an important role in the functioning of the corpus luteum. The corpus luteum is responsible for the production of progesterone, a hormone that acts to regulate the estrus cycle. Therefore, if thalidomide inhibits the production of TNF- α in all areas of the body, this could disrupt ovarian function and be a possible mechanism for how thalidomide disrupts the reproductive function of female humans. Future studies devoted to this hypothesis would be quite interesting.

As with all research, there were some problems that were encountered along the way which could affect the validity of this research. First, it's been noted that female mice are very sensitive to the environmental conditions when it comes to their estrus cyclicity. If the cages are too small or if there are too many females in one cage, this can offset the estrus cycle. Although the cages used in this study were on the large side and

there were only five females per cage, this might have played a role in the degree of irregularity seen in the estrus cycles. Second, it is possible that the lavage method of obtaining vaginal cells or the forced feeding of thalidomide stressed the mice out and caused irregular estrus cycles. If the mice were highly stressed, they might increase their production of the steroid hormone cortisol, which could cause irregularities in the cycle. These possible disruptions need to be taken into consideration when analyzing this study. Also, in terms of the digital analysis of the organs to determine organ weight and blood vessel density, it is possible that there are more precise ways of obtaining these measurements. Unfortunately, the software was new to the department and its capabilities had not yet been fully explored. Therefore, future studies similar to this one may need to further consider and find better ways of obtaining similar measurements.

Many people around the world are currently being administered thalidomide to treat leprosy. Even more individuals are involved in clinical trials of thalidomide for the treatment of several types of cancers. As an anti-angiogenic drug, thalidomide would have to be administered chronically, over a long period of time. Because thalidomide has the potential to be used by so many people for a lengthy period of time, it is extremely important that we realize what effects thalidomide might have on the healthy systems of the body such as the reproductive system. Even though those processes of the body may be deemed less important when considering the overall diseased state of the patient, it is still essential to fully comprehend every aspect of the drug's functioning inside the person in order to compensate, if necessary, for deleterious side effects of thalidomide treatment.

Over 60 angiogenesis inhibitors are currently on the market or being studied through clinical trials (Marx, 2003). Although angiogenesis inhibitors differ in the factors of the body that they target in order to inhibit angiogenesis, all of these factors are endogenous to the body, meaning that they most likely have important functions in maintaining the overall health of the person. Thus, it is important that we understand how angiogenesis inhibitors may affect normal processes in the body that involve angiogenesis. Once we are aware of possible side effects, we can weigh the consequences and make more informed decisions concerning treatment of diseases using anti-angiogenesis therapy.

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