

Specific Aims

According to the results of numerous research studies, DNA hypermethylation is linked to the development of clear cell renal cell carcinomas (ccRCC) [1]. It downregulates cell cycle control and DNA damage repair genes and upregulates tumor cell invasion and metastasis by activating oncogenes in renal cells [2]. The main factor that causes ccRCC, however, is the hypermethylation of the promoter and enhancer regions in CpG islands, which can result in the inactivation of important tumor suppressor genes in clear cell renal cells known as Von Hippel-Lindau (VHL) [3]. Therefore, DNA hypermethylation is essentially involved in the transcriptional silencing of these genes. The silencing of the VHL tumor suppressor genes is caused by an excessive number of methyl groups blocking the genes from transcription factors, which results in an absence of tumor suppressor proteins and therefore contributes to carcinogenesis.

I hypothesize that, with the use of hypomethylating agents, ccRCC could be cured or prevented as these agents have been shown to inhibit aberrant cell proliferation and migration of renal cells both in in vitro and xenograft studies. Hypomethylating agents especially play a significant role in reactivating the transcriptional activity of VHL tumor suppressor genes, which is a key reversal mechanism that can overturn the effects of hypermethylation in clear cell renal cells. As a key part of my research, I will also be investigating whether hypomethylating agents are effective in demethylating important H3K4me1 marks in kidney-specific enhancer regions to reactivate the transcriptional activity of VHL tumor suppressor genes. Therefore, this proposal aims to investigate whether hypomethylating agents could potentially be used as a valid therapeutic agent against ccRCC.

Specific Aim 1: Investigate the precise epigenetic mechanisms by which hypomethylating agents counteract the effects of hypermethylation in ccRCC.

1a: Determine to what extent and at which precise locations along the gene body hypomethylating agents reactivate VHL tumor suppressor genes by the demethylation of H3K4me1 marks.

1b: Use of hypomethylating agents in the downregulation of overactive Wnt Pathways by reactivation of the Wnt antagonist, WIF-1

1c: Use of RT-qPCR to determine the efficacy of hypomethylating agents in reducing cell cycle-related gene expression by downregulation of the Wnt Pathways.

1d: Assess the difference in the quantity of proteins produced between tumor suppressor and oncogenes with the use of Western Blotting.

Specific Aim 2: Investigate the effects of hypomethylating agents on kidney physiology.

2a: Assess the change in renal cell and overall kidney function after the administration of hypomethylating agents.

2b: Compare and contrast the differences in symptoms and prognostic outcomes between patients receiving a placebo and patients receiving hypomethylating agents as therapy for ccRCC.

Background

A study conducted on the role that alterations in DNA methylation play in ccRCC found that the hypermethylation of promoter regions in numerous tumor suppressor genes either partially or entirely silenced the genes [2]. This hypermethylation results in the addition of many methyl groups to CpG dinucleotides which represses the transcriptional activity of VHL tumor suppressor genes by preventing the binding of transcription factors [2]. Hypermethylation of these tumor suppressor genes in ccRCC is caused by mutations in DNA methyltransferase-1 (DNMT1) enzymes, which have been shown to increase cell proliferation, reduce apoptosis, increase colony formation and invading ability, and enhance cell migration [4]. The study used a HELP (HpaII tiny fragment Enrichment by Ligation-mediated PCR) assay to analyze genome-wide patterns of DNA methylation in ccRCC at a high resolution of 1.3 million CpG nucleotides [2]. The researchers of the study found that most of the hypermethylation occurred in renal-specific enhancer regions of the gene body that were linked to H3K4me1 marks [2]. The chromatin modification of these H3K4me1 marks at the active enhancer regions occurs when they are demethylated [2]. Chromatin modification leads to the transcriptional activation of various genes – including tumor suppressor genes [5]. This means that hypomethylating agents are able to restore the function of VHL tumor suppressor genes by removing methyl groups that are bonded to H3K4me1 marks, which allows for chromatin modification and therefore transcriptional activation of the genes. Furthermore, MOTIF analysis of abnormally hypermethylated enhancer regions revealed enrichment of DNMT1 for binding sites of the transcription factors that are normally activated under hypoxic conditions, namely: AP2a, AHR, HAIRY, ARNT, and HIF-1 [2]. This indicates that dysregulated hypoxia may play a rather important role in signaling pathways that lead to the aforementioned epigenetic changes in ccRCC. The functional importance of this aberrant hypermethylation was demonstrated by selective sensitivity of ccRCC cells to low levels of DNA methyltransferase inhibitors. Many accompanying studies found that key players of the Wnt and TGF-beta pathways, negative cell-cycle regulators, and pro-apoptotic genes have also been shown to be epigenetically silenced by hypermethylation mechanisms in ccRCC [6] – which contributes further to the development and progression of the malignancy.

This proposal is important because it can lead to the development of a valid cure against RCC – a disease that accounts for approximately 4% of all adult malignancies worldwide – via an epigenetic pathway [7]. RCC in stage 1 can be surgically removed [8], but there is currently no plausible cure for this disease in its more advanced stages. Therefore, taking advantage of hypomethylating agents as a therapeutic may finally offer a way out of the disease for patients who are in advanced stages of it. As a developing field in the subject of Medicine, there is much to learn about epigenetics – even more so when there is an opportunity on the line to use an epigenetic mechanism to save lives. Not only can this proposal offer significant benefits for ccRCC and potentially other RCC patients, but it can pave the path towards more research on the development of more therapeutics using components of the epigenome.

It is not entirely clear why hypomethylating agents have been shown to activate the VHL tumor suppressor genes and certain oncogenes simultaneously. The overall effect of these agents is, of course, that of tumor suppression. However, the question is why and how hypomethylating agents are able to demethylate these genes with polar opposite functions. In renal cell carcinomas, VHL tumor suppressor genes are hypermethylated whereas oncogenes are often hypomethylated. Nevertheless, hypomethylating agents have been found to increase the expression of four key oncogenes in particular: Interleukin-8, which stimulates angiogenesis and metastasis; G250, which aids cancer progression by the production of bicarbonate which neutralizes the acidic environment surrounding cancer cells; CYTIP, which assists tumor cells in the evasion of apoptotic cytokines; and HLA-G, which has been found to assist tumor cells in evading immunosurveillance [2]. It is unclear why the hypomethylating agents increased the expression of these four oncogenes specifically while the rest of their demethylation activities took place on VHL tumor suppressor genes (once again, the goal is to reactivate tumor suppression). However, that is a key objective of this research proposal that will be addressed: to determine the differences in the precise molecular mechanisms by which hypomethylating agents act on the binding sites of the four aforementioned tumor promoters and compare them to the mechanisms by which they act on the binding sites of VHL tumor suppressor genes. This information includes, but is not limited to: understanding which types of binding sites these genes contain and their precise locations, the precise affinity of the binding sites to methylating agents (both hypo and hyper), and the size and molecular geometry of the binding sites. By acquiring this valuable information, it will be possible to design the hypomethylating agents in a particular conformation that will only allow them to bind to the binding sites of VHL tumor suppressor genes. This, in turn, will solely enable the demethylation of the tumor suppressor genes.

There has never been a therapeutic developed for renal cell carcinomas based on an epigenetic mechanism. Using these epigenetic players to essentially cure a deadly disease is a novel technique that can serve as the basis for the treatment of hundreds of other epigenetic-based disorders in the future. While treatments such as chemotherapy and immunotherapy exist, they are not as targeted and are generally less effective. In addition to these downsides, they can affect and damage healthy cells along with cancerous cells. The use of hypomethylating agents as a therapeutic against ccRCC offers numerous – and significant – advantages over the conventional therapies used to treat cancer. First, they are designed to demethylate tumor suppressor genes in renal cells specifically. Second, they are generally harmful agents that can not possibly damage other cells – after all, they will only be designed to access the nuclei of renal cells. Finally, the third main advantage is that they can not alter the methylation or transcriptional activity of other types of genes. They are designed to only demethylate hypermethylated VHL tumor suppressor genes. With these benefits, there is no doubt that, if successful, hypomethylating agents will present a much better alternative to current treatment methods.

Approach

Specific Aim 1: Investigate the precise epigenetic mechanisms by which hypomethylating agents counteract the effects of hypermethylation in ccRCC.

The objective of this aim is to determine the precise epigenetic mechanisms by which hypomethylating agents counteract the effects of hypermethylation in clear cell renal cell carcinomas. Part of this aim is research-based, while the other part is application-based. The research aspect encompasses the discovery of how hypomethylating agents recognize and remove methyl groups from VHL tumor suppressor genes in the gene body of the renal cells. When an understanding of the molecular mechanism is acquired, this knowledge can be applied to design hypomethylating agents with specialized methyl binding sites and conformations that allow them to remove methyl groups from VHL tumor suppressor genes and prohibit them from acting upon oncogenes to prevent their unwanted transcriptional activation.

Determine to what extent and at which precise locations along the gene body hypomethylating agents reactivate VHL tumor suppressor genes by the demethylation of H3K4me1 marks.

The mechanism by which hypomethylating agents reactivate tumor suppressor genes silenced by the hypermethylation of enhancer/promoter CpG regions will be determined using Methylated DNA Immunoprecipitation (MeDIP). In this process, DNA will be extracted from the VHL tumor suppressor genes of the clear cell renal cells and purified. The purified DNA will then be

sonicated into multiple smaller fragments (each between 400 and 600 base pairs in length) to improve the resolution during imaging, enhance the efficiency of immunoprecipitation, among other things. To enhance the binding affinity of the antibodies to the methyl groups bound to the DNA, the DNA fragments will be further denatured into single-stranded DNA. After denaturation is performed, the methylated DNA will be incubated with monoclonal 5-methylcytosine (5mC) antibodies at 4° celsius overnight. This allows the monoclonal 5mC antibodies to bind to the methylated DNA. After this occurs, magnetic beads each containing a secondary antibody with high affinity for the primary antibody will be added to the sample, which will be incubated once again. These bead-linked antibodies will bind to the monoclonal antibodies. Then, DNA bound to the antibody complex (methylated DNA) will be isolated from the rest of the DNA with the use of a magnet that separates the antibody complexes from the solution. Following this, several washes will be performed using an IP buffer to remove all the unbound, non-methylated DNA in the sample. An enzyme known as Proteinase K will then digest all the antibodies, leaving only the methylated DNA inside the sample. Finally, the enriched DNA will be purified by a technique known as phenol:chloroform extraction to remove the protein matter inside the sample and will then be precipitated in water to be used later. After MeDIP is performed, PCR will be performed to obtain data of the precise methylation levels across a wide genomic range. This data will be compared before and after the hypomethylating agents are applied to determine precisely how much of the methylation was removed from H3K4me1 marks of the VHL tumor suppressor genes by the hypomethylating agents and at what precise location these demethylation events occurred. This is crucial data that will allow researchers to determine on which CpG enhancer/promoter these agents act on to demethylate hypermethylated tumor suppressor genes in renal cells.

Use of hypomethylating agents in the downregulation of overactive Wnt Pathways by reactivation of the Wnt antagonist, WIF-1.

Hypomethylating agents will be directed to reduce the activity of the Wnt pathway in ccRCC by reactivation of a particular Wnt antagonist. The Wnt pathway consists of a set of crucial signal transduction pathways that work together to regulate cell growth and proliferation, migration, cell fate, among other things. In cancer, however, the Wnt pathways go into overdrive. Meanwhile, Wnt inhibitory factor-1 (WIF-1) is downregulated by promoter methylation, which hinders it from performing its main function of suppressing the activity of molecular players in the Wnt pathway when they overact. The overactive Wnt pathways result in aberrant cell growth and proliferation, migration, avoidance of apoptosis, among other things – which are all characteristic of cancer cells. Methylation of WIF-1 is therefore a key factor that contributes to the development and progression of ccRCC. Hypomethylating agents, however, can offer a solution to this. These agents can work to demethylate WIF-1 to reactivate its inhibitory activity in order to reduce the activity of the overactive Wnt pathways. Therefore, they can inhibit the proliferation and migration of many RCC cell lines, hindering the progression of the carcinoma.

Use of RT-qPCR to determine the efficacy of hypomethylating agents in reducing gene expression by downregulation of the Wnt Pathways.

To examine whether the hypomethylating agents were effective in reducing the activity of the Wnt pathways by the activation of WIF-1, reverse transcription quantitative polymerase chain reaction (RT-qPCR) will be performed. First, mRNA will be extracted from the genes that are affected by WIF-1 – namely, those responsible for cell growth and proliferation, cell migration, and apoptosis – and the enzyme reverse transcriptase performs reverse transcription on the mRNA, converting it into cDNA. The cDNA (which is single-stranded) is then converted into double-stranded DNA (dsDNA) by DNA polymerase II, which adds primers to specific regions of the cDNA and creates a complementary strand. Following this step, the reverse transcriptase enzyme is deactivated and the qPCR process begins for amplification of the RNA. In this process, the dsDNA is denatured by heat (95°C) and separated into two single strands. Next, β -actin primers will bind to the single DNA strands during annealing at a temperature of no lower than 40°C. In the final step, elongation, the primers are further extended by DNA polymerase II, which ultimately results in two copies of the original DNA strand. Finally, these strands will be analyzed with a graph displaying fluorescence intensity corresponding to mRNA expression. RT-qPCR will be performed to compare mRNA expression levels before and after the administration of hypomethylating agents in order to determine the efficacy of the hypomethylating agents in reducing the activity of overactive genes influenced by the hyperactivity of the Wnt pathways. The mRNA levels, and thus the level of expression of the genes affected by WIF-1 will also be compared to the β -actin housekeeping gene, which is the internal control of the RT-qPCR test. With this graphical analysis, it is possible to determine whether the hypomethylating agents could be used as plausible molecular players in downregulating overactive cancer-promoting Wnt pathways.

Assess the difference in the quantity of proteins produced between tumor suppressor genes and oncogenes with the use of Western Blotting.

Contrary to their main role of hypomethylating agents in reactivating tumor suppressor genes, they have also been shown to possibly increase the expression of certain oncogenes such as Interleukin-8, HLA-G, G250 and CYTIP. These genes are mainly involved in promoting cell growth, migration and differentiation of tumor cells. When used as a therapeutic agent, the overall effect of hypomethylating agents is that of tumour suppression, however the treatment of ccRCC will improve further when the aforementioned oncogenes are not activated simultaneously with tumor suppressor genes. I will determine the difference in the phenotypic expression of tumor suppressor and oncogene proteins to analyze the difference in the levels of tumor suppressor and tumor promoter proteins using a Western Blot. To do so, I will first collect a sample of tumor suppressor and tumor promoter proteins after administering hypomethylating agents in clear cell renal cells to reactivate the expression of the tumor suppressor and oncogenes. The proteins will be added to different wells and an internal loading control will be

used to ensure that there is an equal loading of the protein samples across all wells. Then, I will separate them by Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to their mass. Following protein separation, an electric current will be applied, forcing the proteins to travel through the gel from the negatively charged electrode to the positively charged electrode. The proteins that are lighter travel further into the gel, whereas the heavier proteins will travel a shorter distance. Following this, the proteins will be blotted onto a sheet made of nitrocellulose or PVDF and each protein will be “stained” with antibodies so that it can be visualized. Specifically, an antibody will bind to each protein and then a fluorescent-labeled secondary antibody will bind to the primary antibody. The fluorescent label will be used to detect the presence of particular proteins and to distinguish between them from each other. Finally, the antibody-protein complexes (visualized as bands with varying thicknesses on a membrane) will be detected by autoradiography and analyzed to assess the quantity of both the tumor suppressor and the tumor promoter proteins in the sample.

Risks: Since the above tests all occur on the molecular level exploring a novel, experimental epigenetic mechanism, the risk for errors in imaging and analyzing the precise locations at which genes are expressed through RT-qPCR and the different proteins that are involved through Western Blotting does exist inevitably. These errors may result in a misinterpretation of how hypomethylating agents precisely interact with hypermethylated tumor suppressor genes and oncogenes, which would then most likely require further testing and investigation into the mechanism. To ensure that this does not occur in RT-qPCR and Western Blotting, the cross-contamination of samples will be avoided, a well-sterilized environment with well-sterilized lab equipment (such as pipettes, centrifuges, vortex mixers, etc) will be ensured, highly effective primers and quality membranes will be implemented, and most importantly, the gold standard of RNA and protein detection devices will be used. Although these actions will not guarantee a perfect outcome in experimental results, they will reduce the chances of experimental errors as much as possible.

Specific Aim 2: Investigate the effects of hypomethylating agents on kidney physiology.

The main objective of this aim is to examine the degree to which hypomethylating agents improve renal function after being administered. Hypothetically, with the administration of more hypomethylating agents, the carcinoma will be hindered from progressing to more advanced stages and may begin to regress. By the demethylation (and thus reactivation) of the H3K4me1 marks of VHL tumor suppressor genes and the Wnt inhibitory factor-1 in renal cells, the expression of tumor suppressors will increase significantly. Therefore, with time, the reduced malignancy of the renal cell carcinoma as a result of the substantial increase in tumor suppressors will lead to enhanced kidney physiology and therefore a remarkable boost in the patient's overall quality of life as well as their lifespan. This theory will be tested on human subjects with ccRCC.

Assess the change in renal cell and overall kidney function after the administration of hypomethylating agents.

Multiple factors will be assessed to determine whether and to what extent kidney function improved after the administration of hypomethylating agents. The first factor that will be assessed is filtration. Filtration rate and volume will be analyzed to determine how efficiently and quickly the kidneys filter out waste that passes through the renal system. This data will be collected using a blood test that measures how much blood the kidneys filter out each minute, known as the Glomerular Filtration Rate (GFR). Meanwhile, a urine test will determine filtration volume and analyze changes in the composition and overall volume of the waste that is filtered out by the kidneys. This will determine whether waste is filtered from the blood and excreted properly by the renal system to assess to what extent renal cell function has been restored after the administration of hypomethylating agents. The next factor that will be analyzed is ion homeostasis. The levels of different electrolytes will be measured by a basic metabolic panel, which would reveal to what extent electrolyte imbalances have been resolved in the blood after administering hypomethylating agents. Finally, blood pressure will be monitored using a sphygmomanometer to assess whether there were any improvements in blood pressure levels and their variability. Together, each of these three factors will be closely monitored in ccRCC patients who receive hypomethylating agents as treatment for several weeks following the administration of the therapeutic to assess the degree to which renal cell and kidney function improved.

Compare and contrast the differences in symptoms and prognostic outcomes between patients receiving a placebo and patients receiving hypomethylating agents as therapy for ccRCC.

Clear cell renal cell carcinoma patients will be divided into two different groups. One will receive a placebo and the other will receive hypomethylating agents for treatment. The patients receiving the placebo will be told that they are receiving a sugar pill that is intended to alleviate certain symptoms and improve the general course of action of their ccRCC disease – although the sugar pill does not actually have any active anti-cancer properties. Therefore, the patients will be psychologically "tricked" into thinking that their symptoms are improving overtime. Although this does not typically improve the actual physiology of renal cells or any of their functions at the molecular level, it may still change the course of their illness and therefore result in an improvement of symptoms. The other groups of patients who receive the hypomethylating agents – which have a plausible therapeutic value – are expected to report a greater improvement in symptoms than those receiving the placebo. What is interesting is whether the placebo will actually improve the prognosis of their disease—despite not improving any physiological function of the renal cells on the molecular level. This is something that will be

investigated in my study. We will monitor several patients for months or years after they receive their treatment — either the placebo or the hypomethylating agents — and compare their prognostic outcomes. This is intended to evaluate how much more effective the hypomethylating agents were in improving prognostic outcome compared to a sugar pill that essentially acts as the control group.

Risks: Monitor for any potential side effects in patients who receive the hypomethylating agents as therapy. Although the chances are low, certain ccRCC patients may have an immune reaction against hypomethylating agents once administered into their body. This may be caused by genetic factors or other health conditions they have. However, regardless of the cause, patients must be monitored for at least a week following the administration of hypomethylating agents to ensure that they are cleared of any potentially life-threatening side effects. Hypomethylating agents will be administered as a therapeutic to a diverse range of people with a diverse range of ages and other health conditions. Each one will be examined to determine how they respond to the therapeutic and record any symptoms they exhibit as side effects. By having a record of the different side effects that patients exhibited in different age groups and with different medical conditions, the optimal dosage at which hypomethylating agents should be administered can be determined. This optimal dosage will most likely differ for different types of patients, however it is the best way to ensure the reversal of epigenetic changes induced by hypermethylation on VHL tumor suppressor genes in renal cells while simultaneously reducing the chances of potentially life-threatening side effects.

Citations:

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