Prolyl Hydroxylase Domain Proteins: A Potential Therapeutic Target for Ischaemic Stroke?

Background

Ischaemic stroke is one of the leading causes of death and disability worldwide. Current treatments are not effective enough to treat ischaemic stroke patients, nor do they promote proper recovery. Developing a drug that fosters an adequate environment for regeneration and repair of neurons following ischaemic damage is therefore essential for improving the prognosis of stroke patients.

Aim

The aim of the article is to evaluate whether prolyl hydroxylase domain protein (PHD) inhibitors could be a potential therapeutic target for ischaemic stroke.

Pharmacology

PHDs mark hypoxia-inducible factor (HIF) to be ubiquitinated under homeostatic conditions. PHDs are incredibly oxygen sensitive and will be inhibited by the lack of oxygen during ischaemia, but following reperfusion, HIFs will continue to be ubiquitinated. Developing a PHD inhibitor for ischaemic stroke treatment would allow HIFs to remain active and promote angiogenesis and erythropoiesis in the brain. Theoretically, this would create an optimal environment for repair and regeneration of neurons. The drug would ideally be a competitive reversible inhibitor of the active site, however, tailoring the drug to be specific to the target will be a challenge. Another challenge will be in crossing the blood-brain-barrier (BBB); however, intranasal administration could be a possible solution. To validate the target, knockout (KO) and mass spectrometry proteomics will be performed.

Conclusion

In summary, the proposed drug has potential to be a therapeutic target for stroke recovery. Unfortunately, treatments for all brain diseases are frequently limited by the BBB and require extensive investigation.

Background

Ischaemic stroke is a cerebrovascular disease caused by an occlusion of an artery that supplies the brain with blood and is one of the leading causes of death and disability worldwide (1). The occlusion is often caused by a plaque/clot in an artery or an embolism that has travelled through the bloodstream to the brain. The blockage prevents the normal supply of oxygen for neurons and glial cells causing them to die within minutes of ischaemia (2). The lack of oxygen not only leads to cell death at the ischaemic core, but also leads to inflammatory and metabolic damage in the surrounding area called the penumbra (2). Therefore, rescuing salvageable brain cells and minimising the damage is just as important as removing the occlusion for patient recovery. Unfortunately, even if a patient survives the acute phase of stroke, the recovery period has a poor prognosis leaving most patients unable to completely take care of themselves and are at greater risk for recurrent stroke (2). While current pharmaceutical treatments for stroke recovery can increase chances of patient survival, it is undeniable that further research is needed for better treatments.

When treating the acute phase of stroke, the two common standard treatments are mechanical thrombectomy (MT) and tissue plasminogen activator (tPA). MT is a procedure that utilizes stent retrievers (SR) or direct aspiration catheters (DAC) whereas tPA is a thrombolytic agent administered intravenously which activates plasmin to dissolve the clot seen in Figure 1 (3, 4). While tPA is theoretically a great option for the removal of an occlusion in a blood vessel, it is known to increase the risk of unwanted bleeding. Furthermore, tPA has a very narrow therapeutic window and must be administered intravenously within approximately 4.5 hours of onset of cerebral ischaemia (3). In contrast, MT has become increasingly popular for occlusion removal for ischaemic stroke because it has a larger therapeutic window and greater efficacy than tPA, particularly in larger blood vessels and proximal occlusions (3). There have also been many studies comparing SRs with DACs and there is now evidence indicating that DACs may be the superior option as they may cause less damage to the endothelial wall, it is a faster procedure, and it is more cost effective (5). Unfortunately, one of the main downsides to both tPA and MT are that while they remove the occlusion, they do not limit the inflammatory damage caused after reperfusion (3). Moreover, MT is a very invasive procedure. Therefore, it is essential to also investigate pharmacological interventions to minimise ischaemic damage to improve the recovery of the patient.

One therapy that is being studied is stem cell transplantation (3). Stem cell transplants would allow for the replacement of necrotic and apoptotic neurons and create a good environment for restoration due to their protein and cytokine secretions (3). However, while many studies have shown promising therapeutic results with respect to promoting recovery and reduced damage, one of the main challenges with this potential therapy is the route of administration. While intravenous injection is the least invasive, there are huge difficulties with crossing the blood-brain-barrier (BBB). Similarly, intraarterial injection is also associated with BBB crossing and is additionally associated with increased risk of occlusions from cellular clumping of the stem cells. In contrast, intracranial procedures would solve the BBB crossing issue, however, this would be an incredibly

invasive surgical intervention (6). Therefore, to maintain the benefits of stem cell therapy and minimise the disadvantages and risks, a different approach could be to target a pathway promotes an ideal environment for nerve cell recovery and neurogenesis. The target pathway that will be further described in this article is the Hypoxia-Inducible Factor (HIF) pathway which is known to promote angiogenesis and erythropoiesis in ischaemic stroke (7). It also has overlapping interactions with the Notch pathway which is involved with neurogenesis. However, this pathway will not be considered as a therapeutic target due to its association with tumour growth (8). The aim of this article is to investigate a possible pharmacological target in the HIF pathway for the long-term recovery of ischaemic stroke.

Pharmacological Intervention of Hypoxia-Inducible Factor Pathway

Target Identification and Considerations

Recently, prolyl hydroxylase domain containing protein (PHD) inhibitors have been approved in some countries for the treatment of chronic kidney disease (CKD) and anaemia (9). Now there is a possibility that PHDs could be a therapeutic target for ischaemic stroke recovery (10). PHDs inhibit HIFs via hydroxylation of proline residues which marks them for ubiquitination under normal conditions. HIFs are transcription factors for vascular endothelial growth factor (VEGF), erythropoietin (EPO), and hypoxia response element (HRE) genes which are associated with angiogenesis, erythropoiesis, and other factors required for ischaemic protection (9). Angiogenesis and erythropoiesis are processes that increase blood vessel growth and erythrocyte synthesis which can increase blood flow and oxygen to ischaemic areas. Therefore, inhibition of PHDs could be beneficial for ischaemic stroke recovery by prolonging the response of HIF to hypoxic injury after oxygen levels return to normal. HIFs are also known to be expressed in rodent neural stem cells which indicates an association with neurogenesis; however, further research is required to understand the role of HIFs in human adult neurogenesis (11). Overall, increasing the activity of HIFs using a PHD inhibitor to improve the recovery of ischaemic stroke patients could be a potential therapeutic treatment. However, there are a variety of PHD isoforms which need to be taken into consideration to decide which PHD to target.

Based on previous research, the PHD2 isoform appears to be the most suitable target. In a study by *Chen et. al.* they performed knockouts (KO) of PHD1 and PHD3, and heterozygous deficiency of PHD2 to determine their roles in mice and cerebral ischaemia (12). They could not perform a complete KO of PHD2 because it would have resulted in embryonic defects. Despite this, they found that PHD2 was statistically significant in its regulation of HIF compared to PHD1 and PHD3. Furthermore, they found that the deficiency in PHD2 was sufficient to sustain life whilst also decreasing apoptotic cells in the penumbra, decreasing BBB disturbance, and most importantly, reducing the volume of the ischaemic core 24 hours after cerebral arterial occlusion. For these reasons, PHD2 seems to be the most suitable drug target candidate.

Another factor that must be taken into consideration is the type of inhibitor that should be used against PHDs since their inactivity is also associated with increased nuclear factor kappa B (NF-kB), a transcription factor for pro-inflammatory cytokines (13, 14). Therefore, using a reversible

inhibitor is a more appropriate choice than an irreversible inhibitor to maintain the benefits of HIFs following reperfusion while minimising the increase in NF-kB. However, it should be noted that PHD1 was most strongly associated with NF-kB increases, but it should still be considered for targeting PHD2 (13, 14).

Target Validation and Crossing the Blood-Brain Barrier

To validate PHD2 as the drug target, the KO mice experiments described from the study in the previous section could be performed. This would allow for the confirmation of the results of their study to further validate PHD2 as the most appropriate target. In addition to the KO of each PHD isoform, the PHD2 inhibitor will be administered to the KO mice intravenously or via nasal spray. The PHD1 and PHD3 KO mice who receive the drug will be compared to PHD1 and PHD3 mice who do not receive the drug. If the KO mice who receive the drug are found to have a greater cognitive outcome and reduced ischaemic volume in the brain compared to those who do not receive it, the PHD2 target may be validated. The PHD2 deficient mice who receive the drug will be compared with PHD2 deficient mice who do not receive the drug. If the KO mice do not have significant differences in ischaemic volume in the brain the PHD2 target may be validated. Lastly, the two modes of administration will be compared.

Unfortunately, targeting PHD2 independent of possible binding to PHD1 and PHD3 will be a challenge because the active components are the same across each isoform (15). One could consider an allosteric inhibitor; however, this would cause irreversible binding which is not ideal due to the previously described associations with NF-kB. Therefore, to increase the specificity for PHD2 active site binding, mass spectrometry protein profiling techniques may be a useful tool to identify differences in post-translational modifications in each isoform (16). Using this information, the drug could theoretically be structured preferentially towards PHD2. In the active site of PHD enzymes the most important components are 2-oxoglutarate and iron binding sites. 2-oxoglutarate is the active component of the enzyme that can hydroxylate proline residues on HIF, however, they require iron as a co-factor. This mechanism can be seen visually in Figure 2A.

One of the current issues with PHD inhibitors that are used for other diseases is that they only target 2-oxoglutarate, but there are multiple 2-oxoglutarate dependent enzymes (10). Therefore, inhibiting the iron binding site is also of interest. While there have been recent in vitro studies on the potential of 2-oxoglutarate inhibition in PHDs for ischaemic stroke, further investigation is required (17). There are also previous studies that have shown the possibility of iron-chelators for inhibiting iron from binding to PHDs, however, iron chelation essentially decreases iron levels (18). This could be an issue if a patient has low normal or low iron levels (10). Therefore, finding a compound that blocks the PHD iron binding site could be a better option to avoid decreasing iron concentrations in the body. A visual of the potential mechanism of the PHD inhibitor can be seen in Figure 2B.

The main issue with creating drugs for the brain is crossing the BBB. Drugs and molecules that can diffuse freely across the BBB must be hydrophobic and less than 400 Da (19). Unfortunately, this means the drug can also diffuse quickly into other tissues and filtered quickly by the kidneys (10). Furthermore, since treatment of stroke patients is very time construed, it would be most

beneficial to use a mode of administration that allows the drug to reach the brain quickly. Therefore, as stated previously in this section, both intravenous injection and nasal spray will be tested. Both methods would not undergo first pass metabolism allowing the drug to reach the brain rapidly, however, the nasal spray would likely be the most suitable option for patient use. One study showed that intravenous injection of an iron chelator PHD inhibitor caused hypotension in rats, but intranasal administration was able to reduce the volume of cerebral ischaemia (20). Intranasal delivery will also be much easier for patients to use and is non-invasive in comparison to intravenous injection. Finally, intranasal delivery of drugs to the brain is desirable due to the high absorption capacity of nasal epithelium and olfactory bulb which also helps with surpassing the BBB (21). A visual of the intranasal administration can be seen in Figure 3.

Therapeutic Window, Therapeutic Range, and Toxicological Considerations

In addition to target identification and validation, the therapeutic window must be considered, especially for drugs intended for the brain. The intended therapeutic window for the PHD2 inhibitor for stroke treatment would be for the recovery period. Based on the research for the CKD and anaemia approved PHD inhibitors, short-term usage of a few months may be better than life-long usage. This is because long-term continuous activity of HIFs may be associated with pulmonary hypertension (15). Additionally, the prolonged stimulation of erythropoiesis elevates the risk of thrombosis (10). However, no severe adverse effects have been shown for short-term use.

The therapeutic range is also important to consider. Unfortunately, some studies of PHD inhibitors for stroke have shown that a high concentration may be required to reach a desired effect but it would have potential undesirable consequences in humans (10). If the therapeutic range is low or narrow, it can increase the risk of toxicological effects. There could be risks with dose concentrations in the brain, in the airways, and interactions with cellular and molecular pathways (21). Additionally, there is a lot of enzymatic activity in the nasal mucosa against environmental/polluting particles and xenobiotics which could be a potential issue with the suggested mode of delivery. Lastly, the pH and chemical components of the drug should also be considered to match the pH of the nasal mucosa to avoid irritation of the nose and airways (21).

Reflections on Drug Development

Sustainability, Health, and Environment

In the process of developing any drug, it not only influences the individuals who would benefit from the drug, it will also have an impact environmentally and ethically. The pharmaceutical industry is one of many sectors that must address the United Nations sustainable development goals (SDGs) due to contributions to pollution and climate change (22). For example, the resources used for research and development contributes to a large amount of waste production for a potential drug that may not make it to the market. For drugs that do get approved, production of the drug and packaging will also contribute to waste. Some of the most detrimental waste products are chemical waste products, especially if they are disposed of improperly. Chemical waste can have harmful effects in both marine and terrestrial ecosystems (22). The pollution created by the pharmaceutical industry also negatively impacts the health of the human population. It is

incredibly paradoxical how pharmaceutical companies produce medicines that improve the quality of life for many people while simultaneously negatively impacting human and wildlife health through pollution. Overall, the SDGs that the pharmaceutical industry most obviously impacts are good health and well-being, climate action, life below water, and life on land.

While the pharmaceutical industry does negatively impact some of the SDGs, it also has positive impacts in others. For example, the pharmaceutical industry provides a variety of jobs which then contributes to economic growth and job security. There are also many companies that offer apprenticeships such as GSK and Astrazeneca which contributes to quality education often leading to a good career within the company. Furthermore, Astrazeneca is an example of a pharmaceutical company that has implemented sustainability targets for the company which address some of the issues mentioned previously. The company has overall been able to use new technologies to reduce wastage, use renewable sources of energy, and investing in natural resources to reduce harmful effects on the environment. Therefore, despite the pharmaceutical industry's effects on the environment, there are companies that have proven that it is possible to run a sustainable business practice.

Disease Prevention and Treatment

Despite its negative impacts on the environment, pharmaceutical companies are incredibly important for disease prevention and treatment. For example, at the height of the COVID-19 pandemic, companies such as Pfizer-BioNTech and Moderna invested in research for the development of a vaccine (23). While COVID-19 continues to spread, the vaccines have helped the public population develop an immunity and minimise the severity of symptoms. Unfortunately, there is still a large population of people in developing countries that do not have access to vaccines, including the COVID-19 vaccine (24). However, this is not an issue that pharmaceutical companies can solve on their own, it is a major global health and political issue (24). Overall, this is one of many examples on how the pharmaceutical industry contributes to disease prevention and treatment.

Ethics and Transparency

Within research, both in academia and industry, ethics and transparency are of great importance. Academic researchers and pharmaceutical companies both require approval from ethics committees for their research which can be a lengthy process. Ethical considerations are most significant in animal studies and human clinical trials. One of the most frequently used ethical models are 3 R studies: replacement, reduction, and refinement (25). The 3 Rs are important ethical considerations when developing study plans and grant proposals because they encourage critical thinking for how to either replace or reduce the usage of animals and refine procedures to reduce animal suffering (26). However, the 3 Rs do not have to be limited to animal studies. They could also be applied to sustainability through efforts to reduce waste production, the possibility of using data from previous experiments, and refining experimental procedures to minimise their impact on the environment. Therefore, in the process of developing a drug it should also be considered whether the estimated environmental impact of the development is ethical. Furthermore, open access data sharing and transparency from companies and academic labs should be greatly

emphasised to support sustainable scientific research. By having transparency of data from experiments, particularly failed experiments, it reduces the possibility of other researchers from performing procedures that have been proven to not work. Reducing repetition of failed experiments that could have been prevented by open access data sharing would help in minimising waste production. It also gives the opportunity for other researchers to evaluate the work of their peers to identify where the procedure went wrong and how to improve it.

Conclusion

In summary, ischaemic stroke is one of the leading causes of death and disability worldwide. The current treatments of ischaemic stroke are not sufficient for patient survival and quality of life. One potential therapy would be to inhibit PHDs to increase activity of HIF which will increase angiogenesis and erythropoiesis to promote the survival of neurons experiencing ischaemic damage. Unfortunately, one of the most common issues in drug development for brain diseases is crossing the BBB. One of the proposed solutions to this could be to use intranasal administration because this route bypasses the BBB. However, this could also present issues regarding dose concentrations. Despite the issue of crossing the BBB, PHD inhibitors have proven to be useful in other forms of ischaemia that can present in the body. For example, recently there have been oral PHD inhibitors approved for the treatment of CKD and anaemia.

While there were many issues to consider when developing a PHD inhibitor for ischaemic stroke, there were also issues highlighted for the process of drug development in general. For example, the pharmaceutical industry is a large contributor to air and waste pollution which negatively impacts the health of both humans and wildlife. While there are some companies that have implemented sustainable strategies, there is still a long way to go, especially since the pharmaceutical industry is not the only contributor to climate change. However, when developing a drug, pharmaceutical companies and academic researchers should also evaluate the weight of positive impact a drug could have for society and individuals. To conclude, developing an effective treatment for ischaemic stroke will have a significant positive impact to those who require treatment, however, pharmaceutical companies must implement more sustainable strategies for future research.

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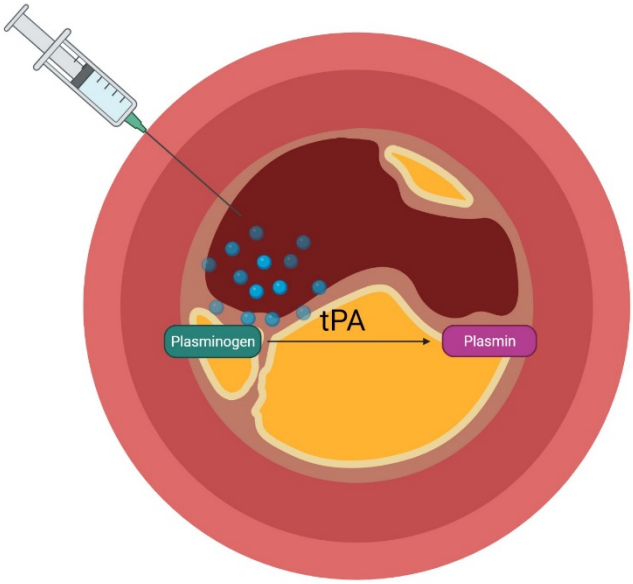

Figure 1: The figure is a visual representation of the most standard treatment for stroke which is tPA. Through intravenous injection of tPA, plasminogen converts to plasmin which can dissolve the occlusion in the cerebral artery. This figure was created in Biorender.com.

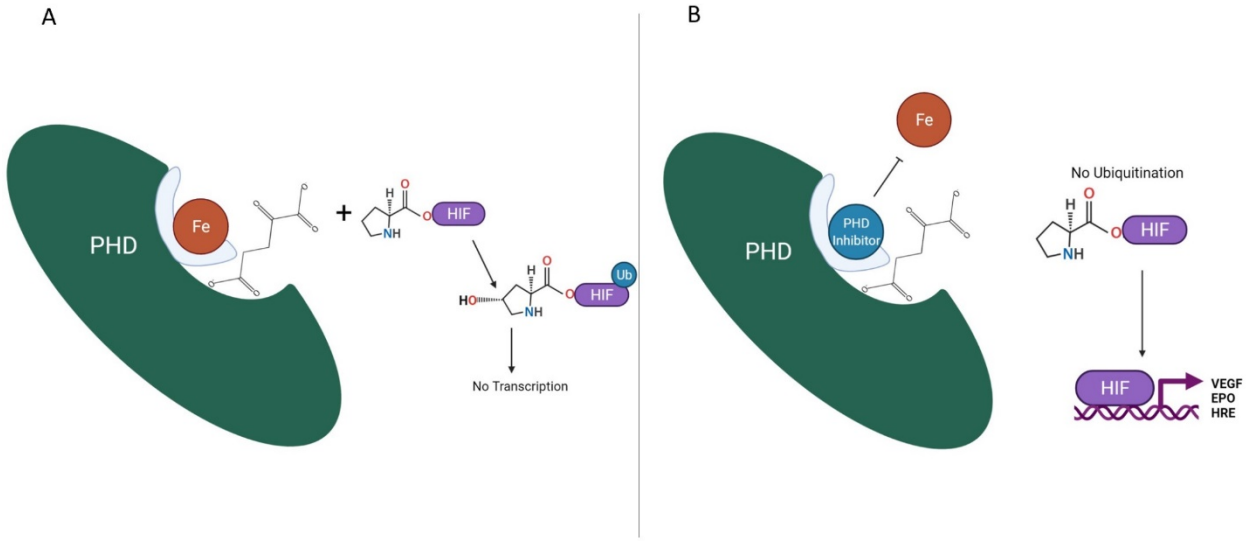

Figure 2: A. The figure is a simplified visual of the function of PHD under homeostatic conditions. In the active site is an iron molecule and 2-oxoglutarate which are responsible for hydroxylating the proline residue on HIF. The hydroxylation marks HIF for ubiquitination which resulting in a lack of transcription of HIF associated genes. **B.** The figure is a simplified visual of the theoretical action of the PHD inhibitor. The inhibitor will block the iron binding site which hinders PHDs

ability to hydroxylate the HIF proline residue. Now HIF can promote the transcription of VEGF, EPO, and HRE. Figure 2A and 2B were created in Biorender.com.

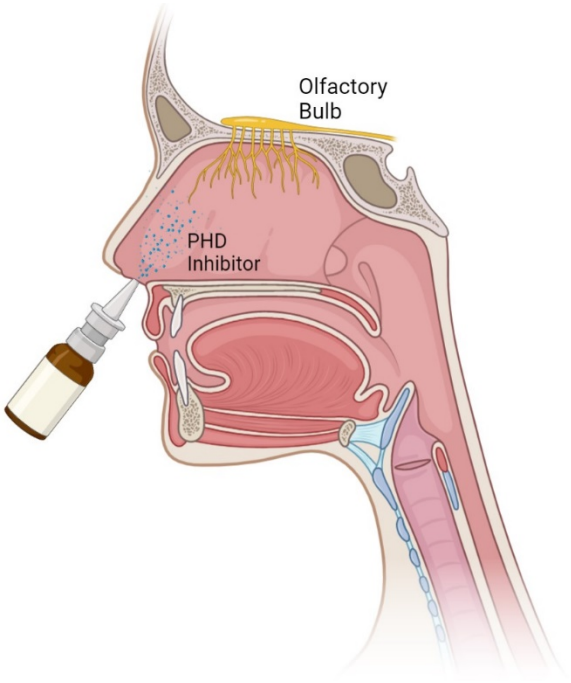

Figure 3: The figure depicts the possible route of administration for the PHD inhibitor which is a nasal spray. The olfactory bulb is visible and extends out of the brain. This gives the PHD inhibitor easier and quicker access to the brain and bypasses the BBB. This figure was created in Biorender.com.