Anti-thrombotic and other pharmacological activity of a Factor Xa inhibiting novel drug/ compound using thrombotic rat models.

Abstract

Stroke has been defined by the World Health Organisation as 'Rapidly developing clinical signs of focal or global disturbance of cerebral function with symptoms lasting 24 hours or longer with no apparent cause other than vascular origin' (Hatano, 1976)

Stroke and thrombotic diseases have been challenging mankind since ages. The disease in itself entails major inconveniences like recurrent hospitalizations, dependency, manifold complications with high mortality rates. Depletion or interrupted oxygen supply to the brain tissue leads to thromboembolic diseases and stroke. Irregularity in the overall homeostatic mechanism with vascular derangement leads to thromboembolic disorders. Since the inception of mankind, many therapeutic drugs have been discovered to curtail the concomitant reactions of a thromboembolic disorder. The antithrombotic and antiplatelet efficacy of the novel chemical compound X will be investigated *in vitro* using platelet accumulation and *in vivo* employing thrombotic mouse models.

Introduction

Types of strokes

Two variations of stroke are evident worldwide namely ischemic and haemorrhagic stroke. When the arterial pathway becomes constricted with a blood clot with to less or no blood supply to the brain tissue leads to ischemic stroke. Ischemic stroke is further classified into embolic and thrombotic depending upon the origin and nature of the clot. Aneurysm of the brain leading to rupture of blood vessels causes haemorrhagic stroke. Akin to ischemic stroke, haemorrhagic stroke is of two types viz., intracerebral haemorrhage and subarachnoid haemorrhage depending on the part of the brain the bleeding occurs. Transient ischemic attack (TIA) is a transitory interruption of blood supply paving chances for more serious stroke attacks. Research has revealed that about 15% of TIA leads to ischaemic attacks.

Prevalence of stroke worldwide

Stroke is the second largest fatal disease and primary cause of adult disability worldwide. A single attack of stroke leaves the victim highly dependent on others. Unlike cancer, the amount spent for stroke every year is very less. For eg. the UK spends 241€ for cancer research in contrast to 48€ on stroke research. Researchers consider that if the minor or transient strokes are promptly treated, about 10,000 spasmodic or recurrent stroke attacks can be evaded (National Stroke Association, 2013)

Epidemiology

In the United Kingdom alone statistics estimates 152,145 stroke attacks per year. Research reveals that about 25 to 33% strokes are recurring. The risk of stroke attack is likely to increase twice every ten years after the age of 55. Statistics has revealed that in the United Kingdom about 26% of strokes attacks people below 65 years old (National Stroke Association, 2013). In general, haemorrhagic strokes are the higher cause of mortality and morbidity in comparison with ischaemic strokes (SSNAP Annual Report, 2014). High concentration of Low Density Lipopolysaccharide (LDL) has been discovered as one of the major risk factor associated with

stroke. Therefore, reduction of LDL and improved levels of High Density Lipopolysaccharide (HDL) have been found to be effective in management of stroke. It has also been observed that the use of statins in cardiac patients have lowered the risk by 25% (Heart Protection Study Collaborative Group, 2002).

Ethnicity

Certain races are also highly vulnerable to the disease mainly because of the genetic makeup and territorial factors. Much research has been conducted to discover the relationship of the disease with ethnic factors. Research has revealed that African and South Asians are highly vulnerable to the disease. Black people are at high risk to develop stroke or thromboembolic disease than the white people (Wang et al., 2003). Similarly, South Asians have higher risk of stroke attack because of the higher percentage of high blood pressure, hyper cholesterol and hyperglycaemia compared to the Westerners. Overconsumption of alcohol and excessive smoking also are regarded as high-risk factors for stroke. About 52% of smokers are likely to be victims of stroke compared to non- smokers. 62% of adolescents have experienced stroke attack during episodes of smoking, drinking and using main line drugs (de los Rios, 2012). The abnormal structural build-up of red cells leads to a condition called sickle cell anaemia. The conventional circle shaped red blood cells change their structure to an abnormal crescent shape leading to sickle cell anaemia.

Patients with sickle cell anaemia have 24% possibility of stroke attack within the age of 45. Similarly, the paediatric population with sickle cell anaemia have a 333% possibility of stroke attack (de Montalembert, (2008).

Stroke research

Although the devastation of stroke is increasing yearly at an alarming rate, research on stroke is still at a snail's pace in comparison to cancer. In UK alone, the annual expense for stroke research has been 56£ in contrast to the 544£ spent on cancer annually.

The commonly used medications for stroke generally fall into four categories: Anticoagulation, Reperfusion, Antiplatelet and neuroprotective.

Antiplatelets

Platelets in the blood produce a compound called thromboxane which is essential for wound healing and stop the flow of blood from an injury. But in stroke patients thromboxane becomes a potential life-threatening compound. Antiplatelet agents inhibit or lower the production of thromboxane. Some of the well known antiplatelet agents are aspirin, dipyridamole, clopidogrel and ticlopidine. It has been observed that there is 5 to 20% of possibility of recurrent stroke after the first TIA. So, antiplatelet agents are prescribed lifelong for patients who have experienced transient or acute ischemic attack (Diener, 2006). Some of the anti-platelet drugs widely used in stroke treatment are aspirin, clopidogrel, dipyridamole and ticlopidine.

Aspirin is a commonly used as an analgesic, anti-inflammatory medicine. It also has excellent antithrombotic activity and are used in patients earlier diagnosed with a stroke attack. Varied doses from 50mg to 325mg/day can be administered based on the severity of the disease. High dose or long-term usage also leads to side effects like gastritis, stomach ulcers and most of all the ability to stop bleeding. Similarly, Clopidogrel is a platelet formation inhibiting drug which

prevents recurrent strokes. Some of side effects of this anti-thrombotic drug are similar to aspirin. Dipyridamole is another widely used anti-platelet drug given as extended release tablets. Dipyridamole inhibits the formation of adenosine which is essential for clotting. In addition, the drug dilates the blood vessels and enables free flow of blood through the blood vessels. Sometimes combinations of aspirin and dipyridamole are administered to achieve good results (Griend et al., 2008).

Combination therapy

Combination therapy offers a positive outlook towards stroke management in the recent years. Many initiatives with combined drugs of different origin and mechanism have been attempted to increase the overall efficacy of the medication. For instance, Aspirin, an antiplatelet drug has greater efficacy when given in combination with dipyridamole or clopidogrel than given as a monotherapy drug. Using this as a base for our research we have attempted to find novel antiplatelet and test their efficacy on animal models as monotherapy drugs or suitable combination drugs.

Factor Xa

Xa is an activated serine protease factor and a crucial enzyme for the coagulation mechanism pathway. The enzyme synthesizes thrombin by forming prothrombinase complex using calcium, phospholipid and the factor Va. Researchers have agreed on the fact that the anticoagulated property can be exerted by inhibiting the function of Xa. Researchers worldwide have discovered Xa inhibiting compounds and natural derivatives which have the anti-thrombotic efficacy. Many of these novel compounds have been tested in different animal models and have proved worthwhile. In our research work we have resolved to discover the antithrombotic efficacy of the novel compound developed in the laboratory. We will be testing on the antithrombotic activity of the compound using venous thrombosis and arterio venous shunt models, template prolongation bleeding time in Sprague Dawley rats and comparing these parameters with synthetically available anti-thrombotic compounds like aspirin, dipyridamole or clopidogrel (Hara et al., 1995).

Materials and methods

Ex-vivo studies

We have proposed to synthesize a novel compound which is a Xa inhibitor and demonstrate its antithrombotic and anti- platelet efficacy of the compound. The compound will be compared with aspirin, dipyridamole and clopidogrel. The antithrombotic activity of the novel compound will be investigated in the venous thrombosis and arterio- venous shunt methods. In addition, the prolongation of the template bleeding time when compared to other synthetic antithrombotic drugs available in the market. To test the efficacy of the drug, Sprague Dawley (SD) rats with body weight ranging from 250- 500g will be employed. To induce thrombosis, the rats will be anaesthetized using pentobarbitone through intraperitoneal injection. Blood wiil be collected from the jugular vein and the drugs will be administered through the femoral vein in the form of bolus. Blood samples will be drawn before and after the administration. Prothrombin time will be measured and coagulation induction will be done using Prothrombin reagent. Activated partial thromboplastin time and anticoagulant activity will be measured will be measured following the protocol as given by Sato et al., 1998. The comparative increase in

coagulation time before and after the administration of the drug will be calculated as the anticoagulation time.

Venous thrombosis in animal models

Venous thrombotic formation method will be done following the method of Reyers et al., 1980 in which the protein content of the thrombus will be measured using photometric method. Arterio-venous shunt model in animals will be performed following the method as given by Sato et al., 1998. The auricle template bleeding time will be done as followed by the method given by MacDonald et al., 1994.

Induction of transient ischemia in animal models

Transient ischemia will be artificially induced in animal models using central cerebral artery occlusion method following the procedure as given by Yang (Yang et al., 1992). The animals will be anesthetized using ketamine and preserved on isoflurane and nitrous oxide. An intraluminal vent will be done on the carotid artery. This vent will produce a focal infarction effect similar to the effects of the human stroke. Ischemia will be maintained for 90 minutes. Following reperfusion after 24 hours the different neurological abnormalities will be recorded. Different stroke parameters like DNA fragmentation of the brain samples by Enzyme Linked Immuno Sorbant Assay (ELISA) will be analysed.

Statistical Analysis

Student's T test, Dunnett multiple comparison test will be employed in each test as required. P values less than 0.05 will be considered as significant.

Conclusion

Even after the evolution of medical knowledge and research, stroke stands third in the fatal disease list in the United Kingdom. It is the leading cause of morbidity, handicap and disability. Antiplatelet is a dual therapy applicable for both ischemic attack and stroke prophylaxis. Antiplatelet medication decreases the possibility of stroke attacks in high risk patients diagnosed with atherosclerosis and cerebrovascular disease. Hence in our laboratory we aspire to identify a novel compound with antiplatelet activity and compare them with the existing synthetic antiplatelets.

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