FORM 2

**THE PATENTS Act 70**

**(39 of 70)**

And

THE PATENT RULES, 2003

**P R O V I S I O N A L S P E C I F I C A T I O N**

(Section 10 and rule 13)

**Title:**

**A Pharmaceutical composition consisting 1,4-di(1H-benzimidazol-2-yl)-1,2,3,4-butanetetrol (BTT) as neuroprotective agent**

Applicant

**Sinoy Sugunan**

**School of Biotechnology,**

**NationalInstitute of Technology Calicut,**

**Kozhikode-673601**

**Kerala State, India**

(An Indian citizen)

The following specification describes the invention.

**FIELD OF INVENTION:**

The present invention relates to pharmaceutical composition of small molecule BTT to block micro RNAs, more preferably to be used for prevention of ischemic brain injuryand recovery from stroke.

**BACKGROUND OF THE INVENTION:**

Ischemic stroke is the second most common cause of death and is the leading cause of long-term neurological disability worldwide. The pathophysiology of stroke involves complex and highly interconnected cascade of cellular and molecular events, leading to the energy depletion, excitotoxicity, oxidative damage, ionic imbalance, calcium overload, loss of mitochondrial membrane potential, inflammation and cell death (Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22:391-397; Sugunan S, Joseph DB, Rajanikant GK (2013) Evolving therapeutic targets in ischemic stroke: a concise review. Curr Drug Targets 14:497-506). The possible pharmacological interventions leading to functional recovery after ischemic stroke are limited. For the above considerations and more, there is a compelling need to expand the narrow repertoire of small molecule neurotherapeutics for this debilitating disease. Drug repurposing has emerged taking advantage of off-target effects of the existing drugs and is a promising, fast, and cost-effective method of targeting ischemic stroke (Fagan SC (2010) Drug repurposing for drug development in stroke. Pharmacotherapy 30:51S-4S).

MiRNAs have been discovered as the regulators of protein expression in various organisms. MiRNAs hybridize to partially complementary binding sequences that are typically localized in the 3' untranslated regions (3'UTR) of target mRNAs, resulting in either cleavage or translational repression in a sequence-specific manner. It is now evident that miRNAs are able to regulate expression of at least one-third of the human genome and play a critical role in a variety of normal biological processes, including cell differentiation, apoptosis, development, and metabolism (Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297; Wienholds E, Plasterk RH (2005) MicroRNA function in animal development. FEBS Lett 579:5911–5922). Furthermore, miRNAs are abundantly expressed in the nervous system and have been initially identified as critical mediators in the regulation of neural development and plasticity (Shi Y, Zhao X, Hsieh J, Wichterle H, Impey S, Banerjee S, Neveu P, Kosik KS (2010) MicroRNA regulation of neural stem cells and neurogenesis. J Neurosci 30:14931–14936). Lately, MiRNAs have been implicated in the etiology of a variety of human diseases including ischemic stroke (Hébert SS, Horré K, Nicolaï L, Bergmans B, Papadopoulou AS, Delacourte A, De Strooper B (2009) MicroRNA regulation of Alzheimer’s amyloid precursor protein expression. Neurobiol Dis 33:422–428; Liu NK, Wang XF, Lu QB, Xu XM (2009) Altered microRNA expression following traumatic spinal cord injury. ExpNeurol 219:424–429; Beveridge NJ, Tooney PA, Carroll AP, Gardiner E, Bowden N, Scott RJ, Tran N, Dedova I, Cairns MJ (2008) Dysregulation of miRNA 181b in the temporal cortex in schizophrenia. Hum Mol Genet 17:1156–1168; Yin KJ, Deng Z, Hamblin M, Xiang Y, Huang H, Zhang J, Jiang X, Wang Y, Chen YE (2010) Peroxisome proliferator-activated receptor delta regulation of miR-15a in ischemia-induced cerebral vascular endothelial injury. J Neurosci 30:6398–6408). Recent studies have shown the involvement of miRNAs in the pathogenesis of ischemic brain injury by using miRNA profiling techniques. These findings suggest several miRNAs as potential candidates for biomarkers or therapeutic targets in stroke (Zhai F, Zhang X, Guan Y, Yang X, Li Y, Song G, Guan L (2012) Expression profiles of microRNAs after focal cerebral ischemia/reperfusion injury in rats. Neural Regen Res 7:917–923). Studies have been carried to investigate the functional significance and molecular mechanisms of individual microRNAs in post-ischemic neuronal death. MiR-497 was reported as a critical player in the regulation of ischemic neuronal death. miR-497 promotes ischemic neuronal death by negatively regulating anti-apoptotic proteins, bcl-2 and bcl-w (Yin KJ, Deng Z, Huang H, Hamblin M, Xie C, Zhang J, Chen YE (2010) miR-497 regulates neuronal death in mouse brain after transient focal cerebral ischemia. Neurobiol Dis 38:17–26). Therefore, inhibition of miR-497 was suggested as a potential neurotherapeutic target to limit ischemic brain injury.

Due to their involvement in stroke etiology, it has become a challenge to develop effective miRNA-inhibiting strategies such as antagomiRs, locked nucleic acids or antisense oligonucleotides. Oligonucleotide-based reagents such as anti-miRNA oligonucleotides provide the most direct route to the inhibition of miRNA function (Naro Y, Thomas M, Stephens MD, Connelly CM, Deiters A (2015) Aryl amide small-molecule inhibitors of microRNA miR-21 function. Bioorg Med Chem Lett 25:4793-4796). Their specificity and efficiency make them excellent tools, however, they pose challenges to therapeutic use. It is mainly due to their poor cellular delivery, and the manifestation of off-target effect ts, such as immune response stimulation and liver toxicity, oligonucleotide-based therapeutics have yet to be translated in to clinical success (Li Z, Rana TM (2014) Therapeutic targeting of microRNAs: current status and future challenges. Nat Rev Drug Disc 13:622-638).

**OBJECTS OF THE INVENTION:**

An objective of the present invention is to provide a pharmaceutical composition of Small-molecule which can inhibit certain miRNA function to prevent ischemic brain injury during or after a stork. The small molecule being 1,4-di(1H-benzimidazol-2-yl)-1,2,3,4-butanetetrol. In comparison to antagomiRs, locked nucleic acids or antisense oligonucleotides Small-molecule is stablein-vivo, is reversible, has a cost effective production, ease of delivery, and enhanced pharmacokinetics.

Another objective of the present invention is to administer a composition of small-molecules that can disturb miRNA function at various steps of miRNA biogenesis and processing.

SUMMARY OF INVENTION

According to one aspect of the invention, there is a pharmaceutical composition of asmall molecule and a pharmaceutically suitable excipient, more particularly1,4-di(1H-benzimidazol-2-yl)-1,2,3,4-butanetetrol (BTT). Pharmaceutical composition is for recovery after ischemic stroke and further prevention.

According to another aspect of the invention, a composition comprising BTT, on administering to a stroke patient inhibits the function of miR-497 and prevents ischemic neuronal death. miR-497 is a micro RNA which is a critical player in the regulation of ischemic neuronal death.

According to another aspect of the invention is a pharmaceutical composition BTT along with other active molecules that can be delivered to a patient in need to prevent an ischemic brain injury.

BRIEF DESCRIPTION OF THE DRAWINGS

Various features and advantages of the present invention should become apparent from the following figures of the accompanying drawings, which demonstrate the process steps we have developed during various process trials carried according to certain preferred embodiment under the invention as well as based on the findings.

Fig 1: Structure of the molecule

Fig 2: Dicer binding region

Fig 3: BTT eliciting significant neuroprotection at 0.05 µM concentration

Fig 4: BTT at 1 µM concentration lowered the cell deaths in hippocampal region.

Fig 5: Levels of miR-497 lowered by 0.05µM of BTT

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a composition comprising a small molecule complex 1,4-di(1H-benzimidazol-2-yl)-1,2,3,4-butanetetrol (as shown in Fig 1) and at least one or more pharmaceutical acceptable excipient suitable of oral administration. The present invention also provides 1,4-di(1H-benzimidazol-2-yl)-1,2,3,4-butanetetrol combined in a suitable excipient for inter venal delivery purpose.

1,4-di(1H-benzimidazol-2-yl)-1,2,3,4-butanetetrol will be referred to as BTT throughout the specification. The composition may also be referred to as the drug throughout the specification if not said otherwise

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| |  |  | | --- | --- | | Molecular Weight | 354.366 g/mol | | XLogP3 | -0.1 | | Hydrogen Bond Donor Count | 6 | | Hydrogen Bond Acceptor Count | 6 | | Rotatable Bond Count | 5 | | Exact Mass | 354.133 g/mol | | Monoisotopic Mass | 354.133 g/mol | | Topological Polar Surface Area | 138 A^2 | | Heavy Atom Count | 26 | | Formal Charge | 0 | | Complexity | 442 | | Isotope Atom Count | 0 | | Defined Atom Stereocenter Count | 0 | | Undefined Atom Stereocenter Count | 4 | | Defined Bond Stereocenter Count | 0 | | Undefined Bond Stereocenter Count | 0 | | Covalently-Bonded Unit Count | 1 | |

Pharmacokinetics

BTTas inhibitors of miRNA function is preferred because; it has stability in vivo, reversibility, cost effective production, ease of delivery, and enhanced pharmacokinetics. Moreover, unlike current oligomer-based tools, which regulate miRNA function through a direct interaction with mature miRNA, small-molecules can disturb miRNA function at various steps of miRNA biogenesis and processing. For example, small-molecules such as BTT could modulate upstream pathways, involved in the transcription of miRNAs, interfere with the miRNA maturation process, or inhibit incorporation of the mature miRNA into RNA-induced silencing complex (RISC).

In the present invention a 3D structure of the hairpin loop region of miR-497 generated for docking based virtual screening BTTshowed the best docking score among other molecules. BTTwas evaluated for neuroprotective efficacy against oxygen-glucose deprivation (OGD) and reoxygenation-induced neuronal cell death in N2A cells (mouse neuroblastoma cells) and mouse organotypic hippocampal slice cultures. BTT lowered miR-497 levels significantly in N2A cells compared to OGD treated cells.

In one embodiment of the invention, administering BTT will prevent post-ischemic neuronal death in a patient who just suffered an ischemic stroke or recovering from one. BTT with its enhanced pharmacokinetics is able to overcome in-vivo barriers and target miRNA function, in this case inhabiting miR-497 to prevent or at lease limit ischemic brain injury.

In one embodiment of the invention, administering BTT will repair the neurological function in a patient suffering from memory loss (or Alzheimer’s disease( Alzheimer's disease is a progressive, degenerative disorder that attacks the brain's nerve cells, or neurons, resulting in loss of memory, thinking and language skills, and behavioral changes. Taken from- Alzheimer's Foundation of America www.alzfdn.org/AboutAlzheimers/definition.html).

**Example 1**

MiRNA 3D structure modelling

The pre-miRNA stem-loop sequence of human miR-497 (hsa-mir-497) was retrieved from miRBase (Accession number: MI0003138). The structure of miRNA was predicted using the MC-Fold and MC-Sym web-based server pipeline. In order to make the modeling process easier, only the miRNA from 26 to 85 nucleotides (60 base pairs) that contains the hairpin loop region was considered for 3D structure prediction. MC-Fold was used to predict the secondary structure of miR-497. It is a knowledge-based type that involves Turner’s thermodynamics tables combined to a nearest neighbor model, which is a most prominent approach to structure prediction . Further, Nucleotide Cyclic Motifs (NCMs) (Parisien M, Major F (2008) The MC-Fold and MC-Sym pipeline infers RNA structure from sequence data. Nature 452:51–55) are used to derive a pseudo-potential energy function that takes the in-stem non-canonical base pairs into account. MC-Fold predicts sub-optimal structures and gives them as output, ranked by energy. The edit option for the best structure was selected to obtain the MC-Sym script.

The MC-Sym script was modified to model the hairpin loop. The fragment and threading libraries were modified to enhance the model prediction. Further, backtracking was carried out separately on upper stem, interior loop and lower stem to assemble the whole miRNA structure. The modified MC-Sym script was given as input to the MC-Sym server to predict the tertiary structure of miR-497. MC-Sym predicts all-atoms RNA tertiary structure from the secondary structure.

The miRNA sequence along with its secondary structure was given as an input to MC-Sym that uses NCM fusion process to generate a list of tertiary structures. The obtained 3D structures were post-processed by relieving them to remove any steric clashes and correct the positions of the backbone atoms. Further, the minimization was carried out on all the models using the Tinker molecular modeling package (Ponder JW, Richards FM (1987) An efficient Newton-like method for molecular mechanics energy minimization of large molecules. J ComputChem 8:1016–1024; Ren P, Ponder JW (2003) Polarizable atomic multipole water model for molecular mechanics simulation. J Phys Chem B 107:5933–5947) with a steepest-descent search on the Amber99 force-field (Wang J, Cieplak P, Kollman PA (2000) How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? J ComputChem 21:1049–1074) Finally, all the miRNA structures were sorted according to their P-Score, which is a pseudo-potential energy function that measures the A-RNA likeliness of the structures. The structure with the best P-Score was selected for further analysis.

Identification of the binding site: The small molecule binding site should be present at the dicer binding region on miR-497. The dicer binding site was predicted using various approaches. The mature miRNA sequences given in MiRBase were compared with the stem-loop sequence to analyze the dicer binding region. In addition, MIRmat(He C, Li YX, Zhang G, Gu Z, Yang R, Li J, Lu ZJ, Zhou ZH, Zhang C, Wang J (2012) MiRmat: mature microRNA sequence prediction. PLoS One 7:e51673) was used to predict the dicer binding site on miRNA. Further, the small molecule binding sites on miRNA were predicted through SiteMap module of Schrodinger (Halgren T (2007) New method for fast and accurate binding-site identification and analysis. ChemBiol Drug Des 69:146–148)

Docking-based virtual screening: In order to study the nature of miRNA binding compounds and to reposition the existing drugs, the FDA approved drug database was used for the structure based screening with the modeled miR-497 structure. Ligand preparation and docking was carried out as described previously (Fayaz SM, Rajanikant GK (2014) Ensemble pharmacophore meets ensemble docking: a novel screening strategy for the identification of RIPK1 inhibitors. J Comput Aided Mol Des 28:779–794). The molecules from FDA drug database, NCI and PDB libraries were subjected to ligand preparation by the LigPrep module of the Schrodinger suite (LigPrepv2.3, Schrodinger, LLC, New York, NY). The ligands were processed to assign the suitable protonation states at physiological pH 7.2 ± 0.2. Conformer generation was carried out with the ConfGen torsional sampling by using OPLS 2005 force field.

Structure-based screening was carried out using the virtual screening wizard of Schrodinger suite. Glide module was used for all the docking protocol using OPLS 2005 force field. It was reported that Glide could predict the most probable binding modes of small molecules with RNA. The default settings as available in the software package were used for the refinement and docking calculations. The Van der Waals radii were scaled using a default scaling factor of 0.80 and default partial cutoff charge of 0.15 to decrease the penalties. The option to output Glide XP descriptor information was chosen (Glide v5.7, Schrodinger, LLC, New York, NY), which deduces energy terms such as H-bond interactions, electrostatic interaction, hydrophobic enclosure, and π-π stacking interactions. Finally, post-docking minimization was implemented to optimize the ligand geometries (refer to Fig 2).

|  |  |
| --- | --- |
| **Molecule** | **Docking Score** |
| 1,4-di(1H-benzimidazol-2-yl)-1,2,3,4-butanetetrol or BTT | -10.9915 |

Table1: Docking score of BTT

**Example 2**

**Neuroprotection assay**

Cell culture

Mouse N2A neuroblastoma cells were maintained in Dulbecco's Modified Eagle Medium: (DMEM) and 10% (vol/vol) heat inactivated foetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, USA).

Oxygen-glucose deprivation (OGD) induction

The cells were divided into control group (normal N2A cells), OGD group (cells were treated with 4 hour OGD followed by 20 hour reperfusion), small molecules + OGD group (the cells were treated with the various concentrations of small moleculesduring OGD and post-OGD stages, and continued till 20 th hour) .

OGD was induced by replacing the culture medium with glucose free OGD medium and the cells were placed in Modular Incubator Chamber (Billups-Rothenberg, Del Mar, USA). The chamber was sealed and flushed with a mixture of 95% N2 and 5% CO2 for 10 minutes with a flow rate of 40 litres per minute. Two ports of the chamber were then sealed and the chamber was placed at 37°C incubator for 4 hours. OGD was terminated by replacing the glucose free OGD medium with normal DMEM medium and returning the culture to the incubator under normoxic conditions (20 hour reperfusion). Control cells were maintained in a normoxic condition (5% CO2 and 95% air) with regular DMEM.

Cell viability

Cell viability was measured at 20 hour post-OGD using the Cell proliferation kit I (MTT) (Roche) with formazan solubilized in solubilizing buffer provided in kit and absorbance determined at 570 nm using a microplate reader (TECAN, Infinite200 pro, Switzerland).

Organotypic hippocampal- slice culture

Preparation of slice culture

All animal experiments were carried out at University of Basel in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and permitted by animal care committee of the Canton of Basel. Cultures were prepared from B6CF1 mice (CB6) as described previously (Chip S, Zhu X, Kapfhammer JP (2014) The analysis of neurovascular remodeling in entorhino-hippocampal organotypic slice cultures. J Vis Exp e52023). Mouse pups were decapitated at postnatal day 4 and their brains were dissected aseptically and the brains were extracted and placed in ice-cold preparation medium medium (minimal essential medium (MEM), 1% GlutaMAX (Gibco, Invitrogen), and pH 7.3). The hippocampus together with the entorhinal cortex (EC) was dissected and 350µm transversal hippocampal slices were sectioned using a McIllwain tissue chopper under aseptic conditions. Slices were separated, transferred on to a permeable membrane (Millicell-CM, Millipore), and incubated with incubation medium (50% MEM, 25% Basal Medium Eagle, 25% horse serum, 1% GlutaMAX, and 0.65% glucose) with 5% CO2 at 37°C. The medium was changed the next day and then every other day up to 1 week.

Oxygen glucose deprivation induction

The slices were divided into control group (normal slices), OGD group (slices were treated with 1 hour OGD followed by 24 hour reperfusion), small molecules + OGD group (the slices were treated with the various concentrations of top hit small molecules during and post OGD stages).

Organotypic hippocampal slice cultures were cultured for 6 days (DIV6) before exposure to hypoxia or OGD. Oxygen and glucose deprivation medium was prepared as detailed by Rytter et al. (Rytter A, Cronberg T, Asztély F, Nemali S, Wieloch T (2003) Mouse hippocampal organotypic tissue cultures exposed to in vitro ‘ischemia’ show selective and delayed CA1 damage that is aggravated by glucose. J Cereb Blood Flow Metab 23:23-33) using Neurobasal medium containing: 2% B27 and 1% glutamax (Life Technologies). Before the slices were exposed to OGD, the OGD medium was equilibrated with N2 for 30minutes in Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA, USA). OGD was induced by replacing the culture medium with glucose free OGD medium and the slices were placed in the above mentioned chamber. The chamber was sealed and flushed with a mixture of 95% N2 and 5% CO2 for 15 minutes. Two ports of the chamber were then sealed and the chamber was placed at 37°C incubator for 1 hour. Slices were treated with different concentrations of amikacin (0, 1, 5 and 20 µM) during OGD and post-OGD stages. OGD was terminated by replacing the glucose free OGD medium with incubation medium and returning the culture to the incubator under normoxic conditions (24 hour reperfusion). For observing OGD induced neuronal death in hippocampal CA1 region propidium iodide (PI) staining was performed. PI was added 30 minutes prior to fixation.Slices were fixed with 4% freshly prepared paraformaldehyde (PFA) and were placed in refrigerator for overnight at 40 C. Stained slices were mounted on glass slides with coverslip using Mowiol. The microscopic observations were made on an Olympus AX-70 microscope equipped with a Spot Insight digital camera. The images were taken with 20x magnification. The neuronal death was quantified.

MiRNA assay

Cells were seeded in 6 well plates at a density of 106cells/well and incubated at 370 C for 24 hours. Cells were treated with top hit molecules during and post-OGD stages.

Total RNA was extracted from the harvested cells, using mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific, USA). Reverse transcription was performed using the TaqMan MiRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). Equal amounts of total RNA (20 ng) were reverse-transcribed with 100 mM dNTPs (with dTTP), 50 U reverse transcriptase, 0.4 U RNase inhibitor, and specific microRNA reverse transcriptase primers at a condition of 16°C for 30 mins, 42°C for 30 mins, and 85°C for 5 mins (Yin KJ, Deng Z, Huang H, Hamblin M, Xie C, Zhang J, Chen YE (2010) miR-497 regulates neuronal death in mouse brain after transient focal cerebral ischemia. Neurobiol Dis 38:17–26)

PCR reactions were then conducted using the TaqMan® MiRNA Assay Kit (Thermo Fisher Scientific, USA) at 95°C for 10 mins, followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min. Each reaction contained 1.33 μl of the RT reaction product, 10 μlTaqMan 2 × Universal PCR Master Mix, 1 μl 20 × TaqMan® MicroRNA Assay reagent in a total volume of 20 μl. The relative microRNA levels were normalized to endogenous sno 202 expression for each sample.

In our studies, we observed that BTT binds to dicer binding region (Fig 1 and Table 1) with good affinity in computational method. Thus, in line with aboveexperiments, the composition BTT exhibited ischemic neuroprotection. Interestingly, expression levels of mature miR-497 were downregulated by BTT (Fig 4), which could be due to inhibition of pre-miRic form. These results suggest that BTT might bind to pre-miR-497 and inhibit its processing into mature miR-497 by the dicer. Further, it might protect the miR-497 target genes (bcl-2) that imparts neuroprotection during ischemic stroke.We have also demonstrated through in vitro post-OGD neuroprotection assay that BTT could protect the cells in nanomolar concentrations. Additionally, amikacin prevented ischemic neuronal death in hippocampal slices (Fig 3).These results highlight BTT as promising neuroprotective agent.

Sinoy Sugunan

Applicant