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The Potential Off-target Neuroprotective Effect of Sister Gliflozins suggest their Repurposing Despite not Crossing the Blood Brain Barrier: from Bioanalytical Assay in Rats into Theory Genesis

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Non-Standard Abbreviations

(**BBB**) Blood Brain Barrier - (**CANA**) Canagliflozin - (**DAPA**) Dapagliflozin - (**EMPA**) Empagliflozin - (**LBTF**) Ligand Based Target Fishing - (**hENT1**) human Equilibrative Nucleoside Transporter 1

Keywords

Dapagliflozin - Drug Repurposing - Gliflozins – Neuroprotective Antidiabetics

Abstract

Gliflozins are successfully marketed antidiabetic agents with a reported neuroprotective effect, this study tests their blood-brain barrier crossing ability. Henceforward, a computational hypothesis interpreting their effects was reasonable after failure to cross into the brain. A chromatographic bioassay for Canagliflozin, Dapagliflozin, and Empagliflozin was developed, validated, and applied to the rat's plasma and rat's brain. HPLC method robustness was tested over two levels using Design of Experiment on MINITAB®. It's the first method for gliflozins' detection in rats' brain tissue. The method was applied on eighteen rats, six for each drug. Concentrations in plasma were determined but neither of them was detected in brain at the described chromatographic conditions. A computational study for the three drugs was endorsing two techniques. Firstly, Ligand Based Target Fishing reveals possible targets for gliflozins. They showed an ability to bind with human Equilibrative Nucleoside Transporter 1, a regulator of adenosine extracellularly. Secondly, a docking study was carried out on this protein receptor. Results showed perfect alignment with a minimum of one hydrogen bond. Dapagliflozin achieved the lowest energy score with two hocking hydrogen bonds. This is proposing gliflozins ability to regulate Equilibrative Nucleoside Transporter 1 receptors in peripheries, elevating the centrally acting neuroprotective adenosine.

1. Introduction

Diabetes Mellitus (DM) and loss of cognitive functions are in a consistently correlated interrelationship. The brain is the most important glucose-dependent organ, and all central cognitive functions are chiefly dependent on insulin: the up regulator of glucose [1]. Accordingly, DM central complications are considered the worst, notably with neurodegenerative impairment [2]. Persistent hyperglycemia leads to an increased incidence of vascular dementia which is common in older people with diabetes [3]. These dementia complications are not related to other metabolic abnormalities, as DM is solely responsible for these resultant cognitive dysfunctions [4] in addition to the known insulin-like growth factor (IGF) signaling mechanisms that are essential in the brain for maintaining synaptic plasticity, and functionality [5].

Controlling DM as a paved path to tackling neurodegeneration is a well-rooted growing belief [1]. Generally, most antidiabetic agents possess a central action either direct or indirect. Those direct-acting antidiabetics function by controlling blood glucose levels leading to adequate glucose supply to the brain. The others act indirectly by increasing central mediators such as gliptins (Dipeptide Peptidase 4 Inhibitors) leading to more incretins that can cross the blood-brain barrier (BBB) and exert central effects [6]. The first quest of this study is to evaluate the BBB crossing ability of three antidiabetics sodium-glucose linked transporter-2 (SGLT-2) inhibitors: Canagliflozin (CANA), Dapagliflozin (DAPA), and Empagliflozin (EMPA) using direct HPLC analysis. Besides, correlating their indirect effects as antidiabetics with their direct effects -if there are any- in the central neurons using computational analysis. This was achieved successfully by developing a new chromatographic bioanalytical method to detect them in rats' plasma and rats' brain homogenate, besides utilizing computational molecular studies searching for their possible other biological targets and executing an interesting molecular docking study.

Some studies showed that some other oral antidiabetics (like metformin & glibenclamide) have a neuroprotective property [7]. A major *Cardiovascular Diabetology* original investigation by Lin *et al* showed that one of SGLT-2 inhibitors, EMPA, ameliorated significantly cerebral superoxide and 8-OHdG - a marker of DNA oxidative damage - experimentally in mice. Moreover, this attenuation of cerebral oxidative stress was associated with reduction of cerebral NADPH oxidase subunit gp91 [8]. Likewise, consolidating this point; EMPA was capable of debilitating neurological defects in rats' model of induced ischemia. Furthermore, levels of hypoxia-inducible

factor 1 (HIF-1) and vascular endothelial growth factor A (VEGF-A) were increased, resulting in decreasing caspase-3 levels. Overexpression of this protein – caspase-3 - plays several roles in Alzheimer's disease pathogenesis involving amyloidosis, formation of neurofibrillary tangles, and neuronal apoptosis [9].

In another study adopting a scopolamine-induced memory loss model for rats; Arafa et al observed the beneficial effect of CANA, which may be correlated with acetylcholinesterase inhibition [10]. A contribution to understanding the role of EMPA in the brain, Hayden et al proved its role in the prevention of neuroglia in diabetic mice [11]. Also exactly like the antidiabetic gliptins, SGLT-2 inhibitors may lead to an increase in GLP-1 concentrations, that in turn can cross the BBB. However, this came with no fine empirical proof that calculates elevations in GLP-1 levels [12].

The gliflozins' (SGLT-2 inhibitors) story has been combined over three centuries. Evoked in 1835 when French chemists isolated Phlorizin as a bicyclic flavonoid from the bark of apple trees. Afterward, in 1886 prominent scientists Josef von Mering and Oscar Minkowski reported its blood glucose-lowering effect and presupposed that the action occurs in the kidneys. This natural flavonoid -Phlorizin- is the progenitor of all contemporary SGLT-2 inhibitors, evinced 130 years later. As once in the early sixties of the twentieth century Chan et al, concluded that Phlorizin blocks glucose transport across the renal tubules. Then in 1972 Glossmann et al, developed unique research on isolated kidney brush border membranes showing that Phlorizin competitively binds with certain receptors in kidney tubules [13]. Those receptors are the same glucose cotransporters proteins responsible for intestinal absorption of sugars. Ten years after, it was discovered by American researchers at the national health institute, that there are differences in glucose transport capacity along the rat kidney tubule: the proximal part of the tubule could absorb more glucose more quickly than the distal part. This effect was attributed later to the presence of SGLT-2. This is depicted in (Figure 1) accompanied by structures of the studied SGLT2 inhibitors.

Over years of intensive and accelerated research more data was revealed, more attention was acquired, and those proteins were of great potential to be a major drug target. The idea that their inhibition will help, originates from the day they were discovered. They chiefly are the only symporters involved in the co-transportation of glucose and sodium in the proximal convoluted tubule of a nephron in diabetic patients.

Today in USA & Europe, there are three approved SGLT-2 inhibitors and currently authorized as new DM second-line treatment; CANA, DAPA, and EMPA [20]. EMPA has been marked as the one with the highest selectivity for SGLT-2 receptors. CANA and DAPA possess some other clinically proven influence. CANA can reduce the chances of stroke and heart attack with an overall decrease in death related to circulatory events in diabetic patients. Reports also, indicate its effect to reduce glucotoxicity and notably improve both insulin sensitivity and beta-cell functions. On the other hand, DAPA decreases the risk of hypoglycemic events and do prevent any unwanted blood pressure drop [14].

Before developing the BBB crossing study for SGLT-2 inhibitors, reviewing literature was mandatory for all the previously developed bioanalytical methods either in human plasma or rats' plasma as described in (Table 1).

The current investigation includes developing a novel HPLC method for SGLT-2 inhibitors' bioanalysis in rats' plasma & brain tissue to check their BBB crossing ability at the time of C_{max}. Spiked samples of blank rats' plasma and rats' brain tissue (with each of the three drugs: CANA, DAPA & EMPA, separately) were analyzed using the proposed LC method. Full-validated calibration curves for each of the drugs were developed. Reasonable lower limits of quantification (LLOQ) were determined as part of the precision and accuracy assessment for each calibration range and considered the lowest measured concentration in the matrix with acceptable accuracy and precision. Successively, the method was effectively applied in concentration quantifications within the biological matrices.

Meanwhile, searching for biological clues explaining how and why gliflozins possess this reported poly-pharmacology and neuroprotective effect. Two techniques of computational analysis were approached. Firstly, in-silico Ligand-Based Target Fishing (LBTF), implementing (CANA-DAPA-EMPA) each time as the query. This is based on 3D and 2D structure similarity search; between the selected query drug and target annotated compound database. The second technique was to proceed with docking in which the most prominent repeated target receptor acquired from target fishing would be docked with gliflozins compound data base including the three investigated CANA, DAPA and EMPA [36].

The results of chromatography study, testing ability of the drugs to pass the BBB along with the computational study would contribute to providing optimal illustration about how these drugs might work off their prescribed targets

2. Methodology

2. 1. HPLC bioanalytical method & BBB crossing study

Instruments

Thermo Fisher Scientific (Massachusetts, USA) UPLC-DAD of a model Ultimate 3000 was implemented using a Hypersil® C_{18} column (100 × 3 mm, 3 μm). Christ® vacuum evaporator (Germany), Acculab® vortex (NY, USA) and Centurion® centrifuge (Sussex, UK) were used for the bioanalytical method.

Chemicals, reagents & biological samples

EMPA, CANA, and DAPA (purity 99.0% ± 1.0), rats' plasma, and rats' brain homogenate were thankfully donated by the Center for Drug Research & Development CDRD (Research entity in faculty of pharmacy the British University in Egypt) based on previous collaborations and research grants. Other HPLC-grade Chemicals (methanol, acetonitrile, potassium dihydrogen phosphate, tertiary butyl ethyl ether, and ethyl acetate were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Ultra-pure deionized water was provided in-house. Blood samples from Sprague Dawley rats of average weight 170-200 g were withdrawn (by CDRD) and centrifuged at 2000 RPM for 20 minutes to acquire plasma. While rats' brain was extracted (by CDRD animal facility), weighed, and homogenized over mortar with pestle via liquid nitrogen. Then this brain homogenate was diluted to the tenth, weight/volume with saline before vigorous vortex for 5 minutes.

The work on animals was ethically approved by the Ethical Committee of the Faculty of Pharmacy, the British University in Egypt, (Approval Number *Ex-2202*). Besides, all the contributing researchers in this study are confirming that all experiments were performed in accordance with relevant guidelines and regulations of the ethical committee in charge. Additionally, all experiments involving animals complied with ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) recommended set of guidelines.

Conditions of the Chromatographic bioassay

The aim of the bioassay was to develop a highly robust technique considering the importance of method supremacy versus complicated matrices of plasma and brain homogenate. The adopted mobile phase was a complex of acetonitrile and methanol with 0.05 M concentration of phosphate buffer in a v/v/v mixture in ratios of (1:1:2) respectively. Phosphate buffer pH was adjusted to 3.5 by orthophosphoric acid. The pump flow rate was 1 mL/min delivering the optimum peak resolution while the injection volume was 10 µL. BDS Hypersil® C18 column with dimensions (100 mm × 3 mm, 3 µm) was utilized, prompting optimal detection, where the column temperature was set stable at 25 °C. The ultraviolet detector was set at 212 nm for CANA, and 225 nm for both DAPA and EMPA.

Biological Samples' extraction Framework

Extraction was done for each 200 µL rats' plasma or rats' brain tissue (after dilution of 10%), where a calculated amount of every drug was added by spiking. Meanwhile, the mixture of the extracting solution made of ethyl acetate and tertiary butyl ethyl ether was prepared (1:1, v/v) and kept cold at -20 °C. From this mixture, 1.2 mL was withdrawn and have been added to each cold sample, sufficiently vortexed for 3 minutes then cooling centrifugation was performed for 15 minutes at 12,000 RPM. Out of the clear upper supernatant, 1.0 mL was accurately withdrawn from each sample and then it was transferred into a labeled Eppendorf tube. These Eppendorf tubes were then transferred into the vacuum concentrator (70 min at 50 °C with 1000 RPM). After complete drying, reconstitution with a mixture of methanol and mobile phase (50:50, v/v), then 3000 RPM vortex stirring was applied for 4 minutes subsequently to mixture addition.

Setting Calibrations and Quality Control Samples

For each drug, 2 mg/mL stock was prepared. Then, nine working solutions (100, 200, 300, 400, 500, 600, 800, 850, and 1000 µg/mL) were prepared in 10 mL volumetric flasks, completing to volume with methanol in all. After that, 10 µL of each flask was added to blank plasma and brain samples (each 990 µL). Subsequently, the obtained concentrations were 1, 2, 3, 4, 5, 6, 8, 8.5, and 10 µg/mL. Four concentrations were selected for Quality Control, which were 1 µg/mL as LLOQ (Lower Limit of Quantitation), 3, 5, and 8.5 µg/mL as "low, mid and high" Quality

Control samples. Each sample underwent the liquid-liquid extraction procedure aforementioned above.

Bioanalytical Method Validation

FDA bioanalytical method validation parameters were adopted and statistically computed [37]. Linearity calculations and calibrations were computed using each peak area. Overall precision (inter-day and intra-day) was studied through the calculation of RSD% for 5 replicates (n=5) that were determined for each quality control (QC) concentration. Accuracy for four QC samples (LLOQ - LOQ - MOQ - HOQ) was quantified expecting no deviation range of more than 15%. Also, extraction recovery was determined by comparing the results of the extracted samples with post-extracted plasma samples possessing the same concentration. The matrix effect as a validation parameter was determined by comparing the peak area of the post-extracted sample with the equivalent neat sample. The last validation step was testing the stability of the QC samples, in which assessments were dependent on comparing the mean percent recovery of samples stored and those prepared freshly before injection with the same concentration. The accepted deviation degree is (±15%) from the optimal concentration except for the LLOQ (±20%). Testing stability: four different conditions were implemented which are: (1) short-term stability referring to analyzing QC samples after being kept for 6 hours at room temperature. (2) Freeze and thaw: that are testing samples after three cycles of -20 °C overnight freezing then putting them to thaw for over 2 hours. (3) Postoperative stability where QC samples are kept in UPLC autosampler at a maintained temperature of 25 °C for a period of 24 hours. (4) Long-term stability testing after 15 days of storage in a -80 °C freezer.

In Vivo Application & the BBB crossing study

The BBB study was implemented using eighteen (n=6) adult male Sprague Dawley rats, six for each drug (weight range 140–180 g and age range 5-8 weeks). They were divided into groups of three one for each drug. Each group was housed in a plastic cage under controlled temperature (22 ± 2) ◦C and 12/12-h dark/light cycle with free access to rat chow and water.

CANA, DAPA, and EMPA were then orally administrated to each rat according to the group, and to ensure the animal survival within the experiment, the doses were assessed according to metabolic conversions from humans to animals. They were calculated after an intensive analysis

of human doses in the marketed tablets and those tried in clinical studies, plus the recently reported rat doses in literature. So, it differs from each drug to the other as follows: 20 mg/kg, 5 mg/kg, and 10 mg/kg for CANA, DAPA, and EMPA; respectively. Then animals were sacrificed according to the reported T_{max}, 2 hours for CANA and EMPA [23] and 1.5 hours for DAPA [38]. Both plasma and brain samples were subjected to extraction before applying the proposed chromatographic method *(section 2. 1. HPLC bioanalytical method & BBB crossing study*.

2. 2. Computational analysis

The notorious multi-pharmacological effects of SGLT-2 inhibitors put roots for their repositioning. In other words, their characteristic clinical features made their possible usage in diseases rather than DM. Searching for possible biological targets is a complicated long pursuit. However, the most recently established bio-/chemo-informatics methodologies are providing efficient support in concluding the most probable target proteins. In this study, in-silico techniques were able to curtail time and effort. Two computational techniques were utilized in this proposed work. Ligand Base Target Fishing (LBTF) in which structure similarity search is carried out between the three drugs and a database of ligands. This technique is reported to be of a wide impact and is highly adequate in hunting accurate targets. The used tool here is SwissTargetPrediction available online http://www.swisstargetprediction.ch/. This is an online free platform provided by The Molecular Modelling group of the University of Lausanne, Switzerland. The adapted structure similarity principle portrays that similar compound molecules are prone to acquire similar properties.

Moreover, in SwissTargetPrediction, it is statistically quantified that similar biologically active molecules are likely to share their target receptors. This estimated quantification validates the authenticity of the "Molecular Similarity Hypothesis" putting forward that 'similar molecules target common proteins' [39]. Subsequently, each of CANA, DAPA, and EMPA was drowned and then used as a query molecule to search among a set of 376,342 actives of defined target receptors (3068 macromolecular targets) (Figure 2). The resultant predicted targets are arranged according to the similarity with ligands through a score that combines 2D and 3D structure similarity with the most similar activity to the query drug. The resultant data for each drug was exported as a spread data sheet for future data analysis. The results of target fishing showed common targets with the highest scores directly after their original SGLT receptors. Two main targets were

detected for the three drugs: human Equilibrative Nucleoside Transporter 1 (hENT1) and Adenosine A2A Receptor (A2AR).

The second used computational approach was a molecular docking study in which all SGLT-2 inhibitors including CANA, DAPA, and EMPA besides the other previously synthesized gliflozins, were docked versus the crystal structure of (hENT1). This study was applied to predict the possible binding pose beside the molecular interactions within target pocket amino acids. The docking study included the co-crystalized ligand isolated from the protein structure. Starting with Protein Data Bank, searching for a target, choosing the most recent with relatively higher resolution, which was PDB ID [6OB6] for (hENT1) plus the co-crystallized ligand [6-{[(4-nitrophenyl)methyl]sulfanyl}-9-beta-D-ribofuranosyl-9H-purine] for (hENT1) [40].

Initially, gliflozins were drowned energy minimized via Open Babel [41] using MMFF94 forcefield. Then, utilizing OpenEye modules, ROCS for conformer generation, and Make Receptor for protein preparation. Docking was carried out by the FRED module which docks ligands blindly to predict all possible binding modes, and lastly VIDA for visualization of the results. ChemGauss4 Scores of dockings were concluded besides possible structure protein interactions for further analysis and examination. Docking poses and similarity-based target fishing representation are shown in (Figure 2) [42-47].

Discussion

3. 1. Extraction novelty and chromatographic renovation

The aim of the current work is to experimentally test the presence of SGLT-2 inhibitors in rats' plasma and rats' brain tissue in order to check their ability to cross the BBB. A new chromatographic method was developed, implementing the previously mentioned UPLC instrumentation relying on UV detection (section 2.1 Conditions of the Chromatographic bioassay). The method development included many trials to overcome the matrix's undesirable interferences. Accordingly, extraction procedures were crucial to retrieve the drugs away from the confusing matrix. Liquid-Liquid extraction was adopted [48]. Applying different types of solvents such as methylene chloride, petroleum ether, n-hexanes, ethyl acetate, diethyl ether, and tertiary butyl ethyl ether, in addition to different mixtures of them on cold biological samples. Finally, after the preliminary investigations mentioned above, a mixture of tertiary butyl ethyl ether and

ethyl acetate (1:1, v/v) was the mixture of choice that achieved the most optimal recoveries. The final extraction conditions (as mentioned under section 2.1. Biological samples' extraction framework) required longer vacuum periods during evaporation, hence samples were evaporated for 70 minutes to attain superior dryness. Afterward, reconstitution with 50 µL methanol and 50 µL mobile phase then using 3000 RPM vortex for each sample was sufficient to dissolve the residue prior to injection.

The aim of the described bioassay was to develop a highly robust technique considering the importance of the method supremacy versus complicated matrices of plasma and brain homogenate. During UPLC method development, two stationary phases were tried. C18 Hypersil® Gold column (50 mm×2.1 mm, 1.9 µm) was initially employed, however, it showed no satisfactory results. That might be attributed to its shortness in length with small particle size. This made it inconvenient to produce good resolution away from relatively polar early eluted interferants. When using the C18 BDS Hypersil® column (100 mm × 3 mm, 3 µm), preferable assay results were achieved. The temperature within the column compartment was kept stable at 25 °C to maintain the method's reproducibility.

Different mobile phase mixtures were tried. Simple mobile phases consisting of methanol or acetonitrile with acidified water (0.1 % orthophosphoric acid or acetic acid) were attempted, but they failed to achieve optimum ionization balance between the three compounds (CANA, DAPA, EMPA), and the interfering residual traces extracted from plasma or brain. Thenceforth, more complicated mixtures were prepared by mixing freshly prepared phosphate buffers of two concentrations (0.05 & 0.01 M) and different pHs ranged from 3 to 4.5 with either methanol or acetonitrile. This pH range was optimum relative to each drug's pKa (CANA= 13.34, DAPA= 12.57, and EMPA= 12.57) to yield sufficient ionization. However, still, no optimum results were obtained regarding peak resolution and symmetry. Lastly and decisively, a ternary mixture of water phase phosphate buffer (0.05 M & pH 3.5) accompanied by an organic phase of acetonitrile and methanol (2:1:1, *v/v*), could achieve the matchless maneuver with the optimal peak purity. (Figure 3) portrays combined chromatograms of the three drugs in rats' plasma and rats' brain tissue. The pump flow rate was settled to be 1.0 mL/min delivering optimum peak resolution. An injection volume of 10 µL was enough to get a reasonable peak area. The ultraviolet detector was set up at 212 nm for CANA, and 225 for both DAPA and EMPA according to the maxima of each drug in

absorption spectra, to guarantee minimal matrix interference for each detection. As well as, adopting 3D spectra over the dynamic range of detection (200-400) to distinguish each drug peak apart from matrix interference. This proposed method was employed in the analysis of the spiked samples of rats' brains and plasma prior to calibration curves' construction for each drug in each matrix ranging from 1 to 10 µg/mL then determination of plasma concentrations of the drugs while application in rats.

Application on Rats

Eighteen animals were categorized into three sets, six for each drug (CANA, DAPA, EMPA). To ensure animal survival within the experiment, the orally administered doses were assessed according to metabolic conversions from humans to animals [37]. They were calculated after an intensive analysis of human doses in the marketed tablets and those tried in clinical studies, plus the recently reported rat doses in literature. So, it differs from each drug to the other as follows: 20 mg/kg, 5 mg/kg, and 10 mg/kg for CANA, DAPA, and EMPA; respectively. Doses were optimum, in the median among the marketed dose and clinical trials concentrations. CANA official dose is either 100 mg or 300 mg with a wider range in human clinical studies from (50-1600 mg) [49], DAPA dosage form contains concentrations of 5 mg and 10 mg and in clinical trials dose concentrations were ranging from 0.1 – 500 mg [50] and for EMPA dosage form has 10 or 25 mg concertation while in clinical trials concentrations ranged between 0.5 to 800 mg [51]. After the T_{max} mentioned for each drug, animals were all sacrificed, chipping in brain and plasma. The proposed UPLC method was used to detect CANA, DAPA, and EMPA in both rats 'plasma and rats' brain tissue. The extracted brain samples of the three drugs showed chromatograms like the blank samples suggesting their inability to cross the BBB (Figure 1 in the Supporting Information) even below the LLOQ as there are no peaks at all, at the specified retention times of the drugs suggesting no BBB crossing based on the current limit of detection (LOD) capability.

Validation of the Chromatographic Method

During the detection of each component at its specific lambda max, no cross-interference between matrix residuals and peak signals was obtained. Comparing blank plasma chromatogram and (LLOQ) peak for each component validated method specificity and selectivity. The current method achieved a well-eluted linearity range between 1-10 µg/mL for all drugs based on the AUP of each drug in both rats' plasma and brain tissue.

QC samples were calculated and analyzed with ± 15% deviation in concentration quantification. Accuracy and precision (both inter-day and intraday) results were shown following RSD% and RE% (n=5) for each concentration. Matrix effect and extraction recovery were also concluded. Although the matrix effect is required mainly in MS detection, we preferred to do it in our study to assure the absence of interference of co-eluting matrix components. All these validation results are summarized in (Table 2). Moreover, method capacity versus different stress conditions mentioned before in section (2. 1. HPLC bioanalytical method & BBB crossing study) were examined, showing accepted adequate results, which are found in (Table 1 in Supporting Information).

Robustness of the UPLC method, as one of the validation parameters, was tested over two levels (& one center point) using Design of Experiment (DOE) applying both Pareto charts & Box Behnken design using MINITAB® software (Table 2 & 3 in the Supporting Information). The lower level is referred to as -1, 0 was the center point, and +1 was the higher level of estimation. Three chromatographic influential parameters were chosen as the studied factors. Flow rates at (0.9, 1, 1.1 mL/min), Buffer pHs at (3.4, 3.5, 3.6), and column temperatures at (20, 25, 30 °C) were tested. The results were analyzed based on both the AUP and retention time output results. The results showed no significant difference based on both the Pareto charts (Figures 2-4 in the Supporting Information) and Response surface methodology (RSM) (Figures 5-10 in Supporting Information). The clearest conclusion that confirmed the robustness was the resulting p-values of more than 0.05 which were considered “statistically insignificant”.

3. 2. Computational Target Analysis and Molecular Docking

Referring to (2. 2. Computational analysis) two integrated in-silico simulations were carried out. LBTF: a general scan for the three investigated drugs to interact with the updated targets database. Thereafter, Molecular Docking between them and the consequent most common target

The remarkable finding through target fishing analysis is the interrelation between CANA, DAPA, and EMPA with adenosine, adenosine receptors, and transporters. As for the three, the most common targets were human Equilibrative Nucleoside Transporter 1 (hENT1), Adenosine Receptors (Adora A2A), and Adenosine Kinase (Adk). Chiefly, hENT1 and Adora A2A possess an essential role in modulating adenosine across body organs and most importantly the brain.

Above all, the prevalent extracellular presence of adenosine is physiologically rational regulating various biological functions. However, adenosine precise concentration extracellularly is depending on the balance between its formation, transportation, and elimination. Where's, a cascade of metabolic ATP ectonucleotidases interactions rules its extracellular upregulation and some others suppose its direct release and passage through the cell membrane. On the other hand, adenosine clearance is governed by Adk, with its high affinity to adenosine leading to major flux into cells through plasma membrane-specified transporters. The importance of adenosine is coming out due to its biological protective roles both in physiological and pathophysiological conditions. Almost, all cell types possess at minimum one adenosine receptor subtype. This illustrates its essential role regarding body organ protection and cell regeneration. This protective biological function differs according to the site of action. It may act by increasing blood supply or reducing inflammation leading to its cardioprotective, cerebroprotective, and neuroprotective influences [52].

Both hENT1 and Adora A2A as molecular targets are widely distributed over human organs, achieving their roles in the adenosine regulation cycle in and outside cells. ENT1 is essential for controlling adenosine levels plus its role in nucleoside uptake for DNA and RNA formation. Clinically ENT1 is a target for a class of medications namely adenosine reuptake inhibitors. Those drugs are blocking the action of the nucleoside transporters, which in turn upregulates adenosine outside cells boosting its extracellular clinical effects over its specified P1 receptors. These extracellular effects are discontinued when adenosine is driven back toward the intracellular via ENTs. Normally, activation of A1, A2A, A2B, and A3 (P1 receptors) by forming an active complex with adenosine cascades its inward transportation [53]. So, inhibition of this reuptake would keep the levels of extracellular adenosine relatively adequate to enhance neurotransmission signal cascades executing central neuroprotection effects. Most probably, this is entirely facilitated through cAMP/p-CREB/Bcl2 signaling routes; it was found that activation of cAMP-dependent protein kinase A(PKA) mediates localized inflammation responses and interrupts neuronal cell apoptosis. In addition, ENT1 inhibition would directly improve cerebral blood flow due to an increment in Nitric oxide (NO) production restoring neuroprotection. Also, a previous study on ENT1 inhibition attributed its neuroprotective effect to the reduction in glutamate neurotransmitters that govern neuronal excitability. To all intents and purposes, the point is: antagonizing ENT1 would greatly affect the adenosine levels outside neurons fortifying

its neuroprotective impact [54]. Not only that, ENT1 knock-out mouse models were made to understand its pharmacological effect and portrayed outstanding elevated levels of systemic adenosine in plasma. These animal models are proven to acquire multiorgan protection, especially during ischemia and reperfusion hard times [55].

Molecular docking studies were implemented on the hENT1 receptor supposing that the interaction between database compounds (gliflozins) and the receptor target would be outside the brain. As they have not been able to pass BBB or even have previously reported having any utility to pass whether in human or in an animal study. Consequently, the study is aiming to elucidate and hypothesize how would gliflozins act as an adenosine reuptake receptor modulator in peripheral organs elevating adenosine levels in the plasma and leading to their neuroprotective central desired outcomes. In correspondence, Adora A2A is a direct receptor for adenosine regulation, but its role as neuroprotective depends on the adenosine concentration balance that is entirely achieved over central activation. On the other hand, Adora A2A peripheral activation gives rise to other cytoprotective signal cascades rather than central, such as being cardioprotective or fibroprotective.

Over the proceeding of the docking process, the co-crystalized ligand isolated with the protein was employed to figure out the physical parameters of the target site of action within protein amino acids prior to protein preparation. Subsequently, molecular docking calculations were simulated for the gliflozins database. Docking results showed that all gliflozins were in stable and optimal alignment inside the receptor molecular surface. According to energy scores, DAPA achieved the highest score while the ligand was the eighth. Low energy complexes between the compounds and binding pocket plus Hydrogen bond formation, consolidate the actual ability of those SGLT2 inhibitors to fit and act as ENT1 potential regulators. Moreover, they might inhibit adenosine reuptake, fulfilling the desired pharmacological neuroprotective outcomes. The three drugs: CANA, DAPA, and EMPA were having higher scores than the protein co-crystalized ligand, showing perfect similar orientation. DAPA was able to form two hydrogen bonds between its sugar moiety and two amino acids (Arginine 345 and Asparagine 407). Its perfect compact posture in the pocket validates the strength of the electrostatic interactions versus the surrounding amino acids. The ligand made one hydrogen bond with Glutamine 158 with its central purine moiety. OpenEye® energy scores are absolute values mimicking drug receptors' overall complex

energy, the lower the value the better is fitting. These scores are presented in (Table 3), also hydrogen bonds between DAPA/Ligand versus receptor are shown in (Figure 4)

Conclusion

The proposed UPLC method confirmed the inability of the studied drugs (CANA, DAPA & EMPA) to cross the BBB based on the rats' study that included eighteen animals after the ethical committee approval. The developed method will be beneficial for future bioanalytical studies especially because it is the first method that considered the quantification of the drugs in the rats' brain tissue. Moreover, it is the first unified method that is considered a direct estimation of all three gliflozins in rats' plasma. The developed extraction procedure was optimized to be valid for both the rats' plasma & brain tissue simultaneously based on a new mixture that had been used for the liquid-liquid extraction. The key for the computational study is that the three antidiabetic agents SGLT-2 inhibitors (CANA, DAPA, and EMPA) have a well-established neuroprotective clinical effect, recently considered, and assessed in the literature. LBTF structure similarity study was carried out using SwissTargetPredict, subsequent to biological literature analysis of the target fishing results. It was found that gliflozins have a considerable affinity toward ENT1 receptor which is crucial in adenosine extracellular concentration. A blind Molecular Docking study showed perfect alignment of the prepared database of all synthesized gliflozins with ENT1 crystal structure isolated with high-resolution. DAPA showed the highest score of binding with the lowest energy complex plus two hydrogen bond formations hocking the structure within the amino acids. This study proposes that an SGLT2 inhibitor might be capable of antagonizing ENT1 receptors in peripheral tissues resulting in a high concentration of adenosine in plasma, subsequently relative elevation in its concentration in the brain via its BBB transporters. These increments of the extracellular adenosine in brain compartments are the reason for the prominent cytoprotecting impacts with overall ameliorations in mind and cognitive functions.

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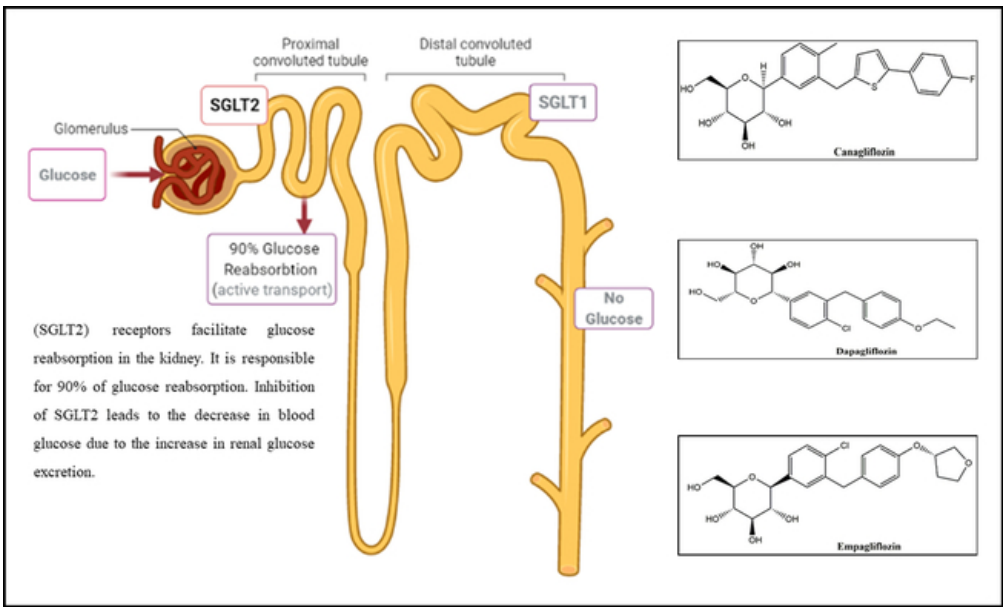
Figure legends

Figure	legend
(Figure 1)	SGLTs effects and nephron distribution plus, CANA, DAPA, and EMPA chemical structures. (Made by www.BioRinder.com).
(**Figure 2)**	SGLT2 inhibitors similarity-based target fishing accompanied with docking results in (A&B) where's, (A) is representing the Highest score DAPA (magenta) plus ligand (green) interactions within hENT1, and (B) is representing Gliflozins (magenta) plus ligand (green) interactions within ENT1
(Figure 3)	LLOQ chromatograms obtained for CANA (1 µg/mL), DAPA (1 µg/mL), and EMPA (1 µg/mL), in plasma (P) and brain (B) samples.
(Figure 4)	(A) Formation of hydrogen bonds (dotted line) between the ligand and GLN 158 within pocket (B) Hydrogen bonds formation between DAPA and (ARG 345-ASN 407)



(Figure 1) SGLTs effects and nephron distribution plus, CANA, DAPA, and EMPA chemical structures. (Made by www.BioRinder.com).

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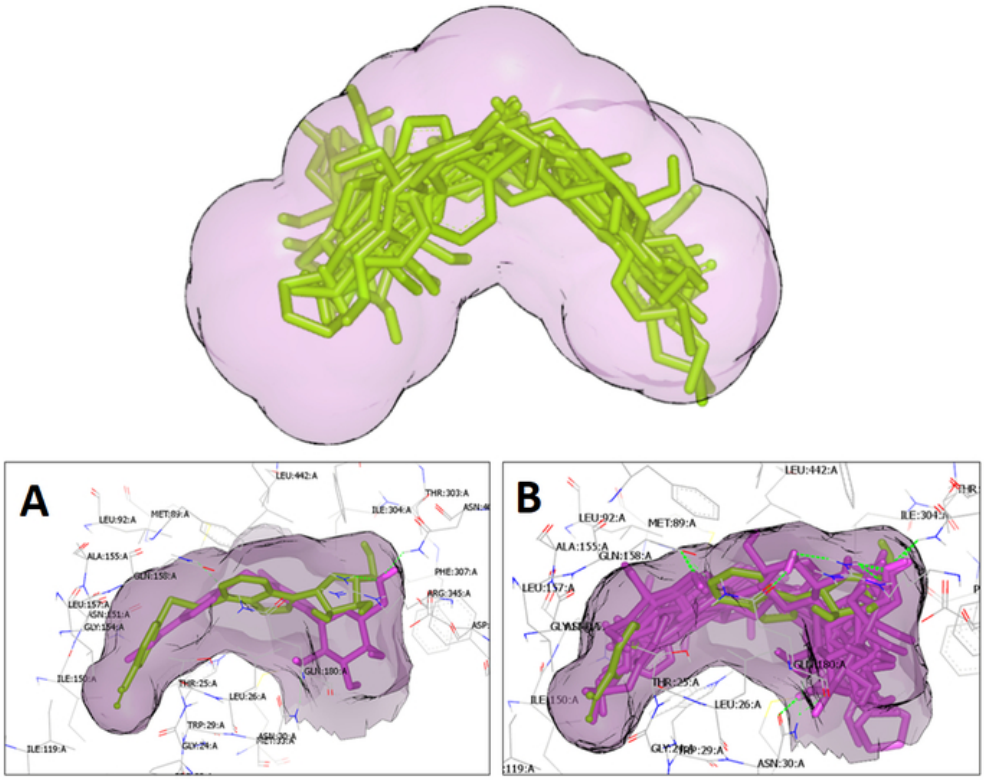

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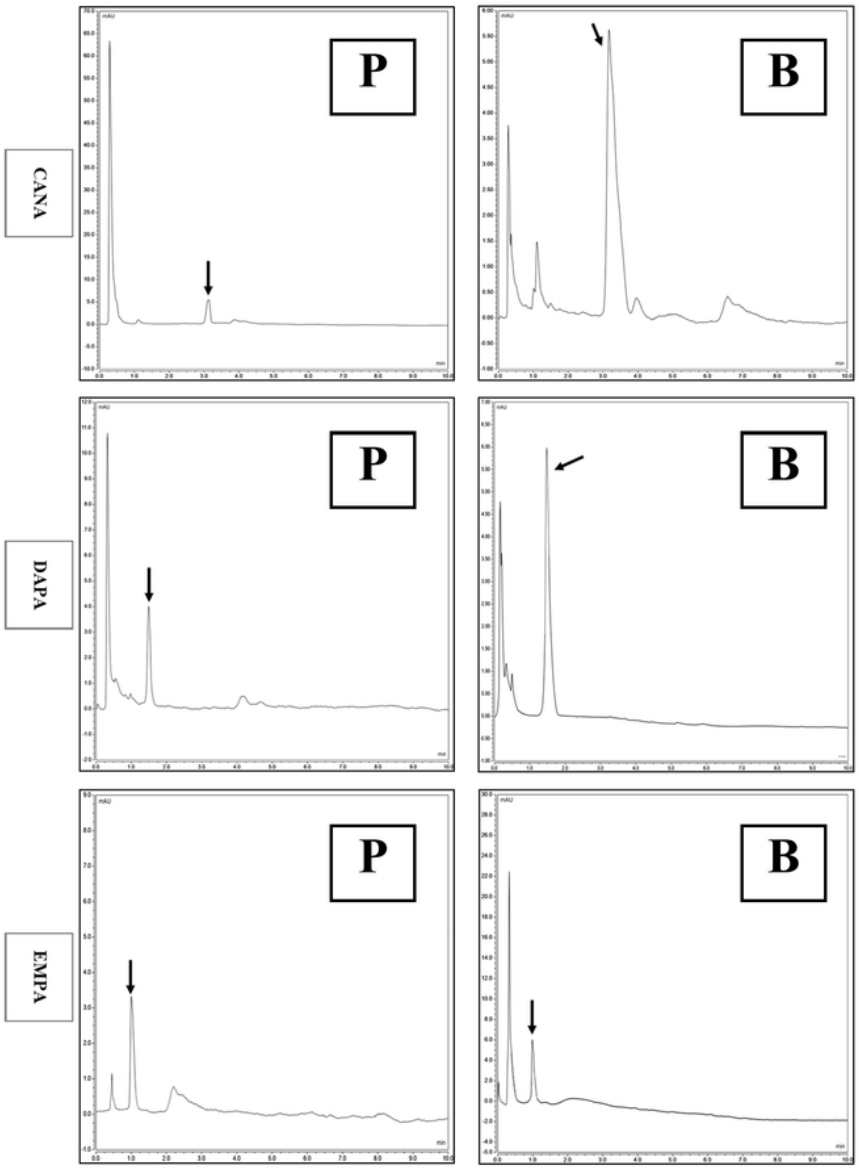
29x23mm (600 x 600 DPI)



(Figure 3) LLOQ chromatograms obtained for CANA (1 µg/mL), DAPA (1 µg/mL), and EMPA (1 µg/mL), in plasma (P) and brain (B) samples.

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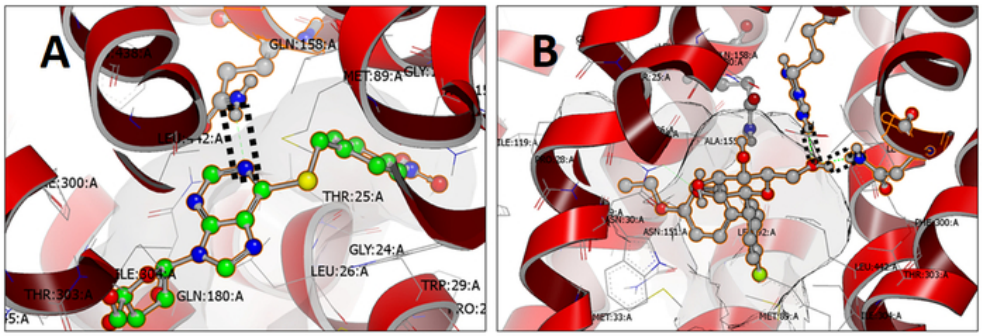

(Figure 4) (A) Formation of hydrogen bonds (dotted line) between the ligand and GLN 158 within pocket (B) Hydrogen bonds formation between DAPA and (ARG 345-ASN 407)

30x10mm (600 x 600 DPI)

(Table 1) Summary of the bioanalytical literature review for SGLT-2 inhibitors

	Author	Year	Matrix	Technique
CANA	*Iqbal et al.* [15]	2015	Rat Plasma	UHPLC-MS/MS
	Iqbal et al. [16]	2015	Human Plasma	HPLC-florescence detector
	Dudhe et al. [24]	2016	Human Plasma	HPLC-UV
	Kobuchi et al. [18]	2016	Rat Plasma	LC-MS/MS
	Dong et al. [19]	2018	Rat Plasma	UPLC-MS/MS
	Mohamed et al. [20]	2019	Human Plasma	LC-MS/MS
	Ramisetti et al. [21]	2019	Human Plasma	LC-MS/MS
	Van Der Beek et al. [22]	2020	Human Plasma & Urine	LC-MS/MS
	Wattamwar et al. [23]	2020	Human Plasma	LC-MS/MS
	Alam et al. [24]	2020	Human Plasma	HPTLC-UV
	Emam et al [25]	2022	Human Plasma	HPTLC-UV
DAPA	*Aubry et al.* [26]	2010	Rat Plasma	LC-MS/MS
	Goday et al. [27]	2018	Human Plasma	LC-MS/MS
	Donepudi et al. [28]	2019	Human Plasma	HPLC-UV
	Omar et al. [29]	2019	Human Plasma	Spectro-fluorimetry
	El-Zaher et al. [30]	2019	Human Plasma	LC-MS/MS
	Van Der Beek et al. [22]	2020	Human Plasma	LC-MS/MS
	Abbas et al. [31]	2020	Rabbit Plasma	TLC-Spectrodensitometry
	Abdel Gawad et al. [32]	2021	Human Plasma	UPLC-UV
EMPA	*Donepudi et al.* [28]	2018	Human Plasma	HPLC-UV
	Mabrouk et al. [33]	2019	Human Plasma	HPLC-DAD
	Shah et al. [34]	2019	Human Plasma	LC-MS/MS
	Wattamwar et al. [23]	2020	Human Plasma	LC-MS/MS
	Omar et al. [29]	2020	Human Plasma	Spectro-fluorimetry
	Rizk et al. [35]	2020	Human Urine	Voltammetry
	Van Der Beek et al. [22]	2020	Human Plasma & Urine	LC-MS/MS

(Table 2) Precision, accuracy, extraction recovery, and matrix effect for CANA, DAPA and EMPA quality control samples.

	Drug	Concentration µg/mL	Intraday precision		Inter-day precision		Matrix effect %	Extraction recovery %
			CV %	Bias	CV %	Bias		
Rat's Plasma	CANA	3	4.94	-8.47	4.07	-4.34	81.25	80.23
		5	0.81	-5.69	4.65	-3.89	86.69	86.33
		8.5	0.49	1.96	1.15	2.83	97.55	94.48
	DAPA	3	1.39	-1.28	0.94	-2.63	80.42	85.75
		5	0.74	-1.31	0.66	-1.53	87.13	89.92
		8.5	1.41	0.59	1.79	1.67	91.73	92.98
	EMPA	3	1.37	-4.44	0.93	-4.56	68.89	65.03
		5	0.42	3.8	0.62	-3.5	87.28	88.84
		8.5	0.93	-1.35	0.65	-1.73	94.36	95.84
Rat's Brain	CANA	3	6.45	7.25	6.34	4.47	80.06	64.47
		5	0.57	-5.63	0.66	-5.08	84.32	69.34
		8.5	2.94	-2.93	0.73	-2.16	90.56	70.54
	DAPA	3	4.98	-13.37	2.24	-13.98	80.23	80.39
		5	5.53	-9.88	1.09	-11.67	86.19	85.29
		8.5	3.63	-8.99	3.12	-9.37	90.90	89.17
	EMPA	3	1.02	-8.81	1.84	-8.09	61.16	62.16
		5	0.81	-5.65	0.92	-5.46	7756	83.31
		8.5	2.5	-3.61	1.87	-3.18	86.04	94.77

(Table 3) Gliflozins scores over ENT1 blind docking

hENT1 Docking

PDB ID [6OB6]

Compounds	**FRED Chemgauss4 score**
Dapagliflozin	-14.5338
Luseogliflozin	-14.8904
Canagliflozin	-14.4942
Sergliflozin etabonate	-14.3366
Ipragliflozin	-14.3048
Empagliflozin	-13.595
Ertugliflozin	-13.2643
Ligand	-13.1777
Tofogliflozin	-13.0448
Sotagliflozin	-12.7628
Remogliflozin	-11.7353