Title:

The bis-(1,2,3,4-tetrahydroisoquinoline) alkaloids Cepharanthine and Berbamine are ligands of SK channels

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Abstract:

Cepharanthine, a multi-target alkaloid which has recently shown to be effective against SARS-Cov-2, and berbamine, an alkaloid characterized as a calcium channel blocker, both share key structural elements with known SK channel blockers. These structural similarities led us to evaluate their affinity for SK channels. Therefore, we performed *in vitro* binding on SK2 and SK3 subtypes and highlighted micromolar to sub-micromolar affinities. Respectively, the Ki values on SK2 and SK3 are 1,318 µM and 1,091 µM for cepharanthine, and 0,284 µM and 0,679 µM for berbamine. These newfound affinities correspond to the concentrations at which the alkaloids are found active against several pathologies. As SK interactions occur at the same levels as their therapeutic effects, there is a strong incentive to further investigate whether SK channels are involved in their pharmaceutical potency.

Keywords:

SK channel, Alkaloid, Cepharanthine, Berbamine, *in vitro* affinity binding, Pharmacology, Cancer therapy

Manuscript Body:

Small conductance calcium-activated potassium (SK) channels, as indicated by their name, are channels permeable to K^+ ions that have a low conductance in comparison to other K^+ ion channels. They are insensitive to voltage and are only activated by an increase in intracellular calcium. Three subtypes are known for this family, namely SK1, SK2 and SK3, and are widely distributed in humans (1). They are mainly found in neurons and other excitable cells where they are involved in diverse physiological and pathological mechanisms such as the regulation of neuronal excitability (2). One subtype can also be overexpressed compared to the others (3) physiologically or depending on special biological contexts, and oftentimes, a pathological mechanism is specifically linked to one of them. The main examples are depression and other mood disorders where SK3 channels are overexpressed in serotonin producing neurons of the basal nuclei (4,5) or atrial fibrillation, where SK2 overexpression occurs (6). Both subtypes are also overrepresented during the evolution of different types of cancer (7-13). The SK2 subtype is found under hypoxic conditions, for example in melanomas (11), while the SK3 subtype is responsible for the mobility of certain cancer cells (7).

The different implications of SK channels make them a target of great interest in therapeutics. Thus, modulators such as extracellular blockers of SK channels are sought after in order to explore their pharmacological functions thoroughly, and to lead the way to drug development projects as novel treatment options for different pathologies. However, even though several very potent blockers, both peptidic and non-peptidic, are known, none displays the necessary qualities to fulfill these roles (i.e. subtype selectivity, SK specificity, high therapeutic index and good druggability). The most prominent of these blockers is the bee-venom toxin apamin. Apamin is a small, 18-amino-acid peptide with 2 disulfide bridges (Fig. 1) that put it in a globular conformation and confer it great stability. It is composed of a short alpha-helix, from amino-acid 9 to 16, and of a beta-turn between amino-acids 2 and 5.

Apamin is a specific ligand of the SK family, and has an extremely high affinity for all three subtypes, in the nanomolar to subnanomolar range, with no effective subtype selectivity. More precisely, its blocking activity, in order, is stronger on SK2 with an IC_{50} of 87.7 pM, then on SK3 with an IC_{50} of 2.3 nM, and finally on SK1 channels with an IC_{50} of 4.1 nM. (14-16) Apamin is therefore regarded as the blocker of reference for the SK family. The site of interaction between the channel and ligand is referred to as the apamin binding site of SK channels. Although apamin was the first ligand to be identified for SK channels and has been known for a long time (14,15), it still interests the scientific community to this day (16).

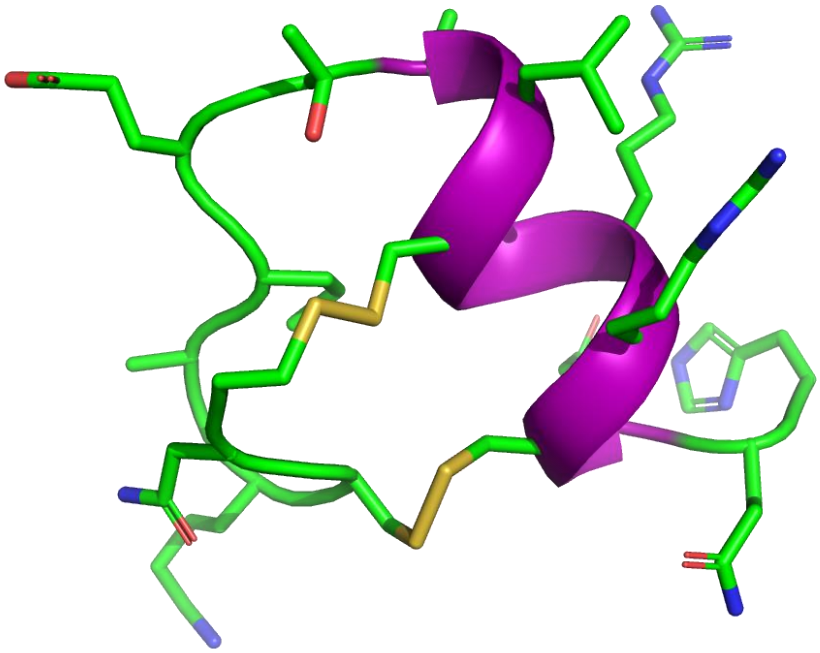

Fig 1. 3D structure and primary sequence of the 18 amino-acid peptide apamin, the reference ligand for the SK family. On the model, the carbon backbone is represented in green, the alpha helix is modeled in purple, and disulfide bridges in yellow sticks. In the sequence, cysteine participating in disulfide bonds are highlighted in orange, the disulfide bonds themselves are represented as connecting lines. The “RXCQ box”, the part of the structure responsible for interaction with SK channels according to current pharmacophore (17-18), is underlined and highlighted in blue. The final amino acid, Histidine, highlighted in red, is amidated. It is also the labeling site of the radio-labeled apamine.

In this pharmacological context, new molecules are investigated for their ability to block SK channels, notably by using a pharmacophore model (17,18) built on the basis of the similarities between apamin's spatial disposition and that of UCL1684 (19), one of the strongest non-peptidic blockers (Fig. 2). Indeed, potent SK blockers typically bear two positively charged nitrogens separated by a comparable distance. Among them, isoquinoline-derived moieties seem to be of great interest (i.e. the isoquinoline heterocycle itself, the partially reduced heterocycle TetraHydroIsoQuinoline (THIQ), and their quaternary analogs isoquinolinium and tetrahydroisoquinolinium) (17). To name a few blockers bearing these scaffolds, curare drugs, such as tubocurarine, were shown to have blocking potencies on SK channels in the micromolar range (20); AG525E1 (21), a molecule derived from the *N*-Methyl-Laudanosine structure (22), also shows submicromolar affinities for the Apamin Binding Site of SK channels subtypes while corresponding to the pharmacophore due to basic nitrogens that bear positive charges at physiological pH (Fig. 2). Moreover, tetrahydroisoquinoliniums and Isoquinoliniums analogs of the compound were also studied (23). In this evaluation, one alkaloid, tetrandrine, a calcium-channel blocker, also revealed a high affinity for the SK family (Fig. 2). This affinity was further reinforced for its bis-TetraHydroIsoQuinolinium analog N-Methyl Tetrandrine, which bears permanent positive charges on the nitrogens of interest. (23)

In this work centered on THIQ derivatives, we investigate two other well-known drugs bearing this scaffold, namely cepharanthine, an antiinflammatory and anticancer drug which was recently considered as a potential small-molecule treatment of COVID19 (24-27), and berbamine, a calcium channel blocker also used as an adjunct in cancer treatments (Fig. 3) (28-29). These two compounds were selected as they correspond to our definition of a potential SK channel blocker, while their mode of action remains unclear for several pharmaceutical indications.

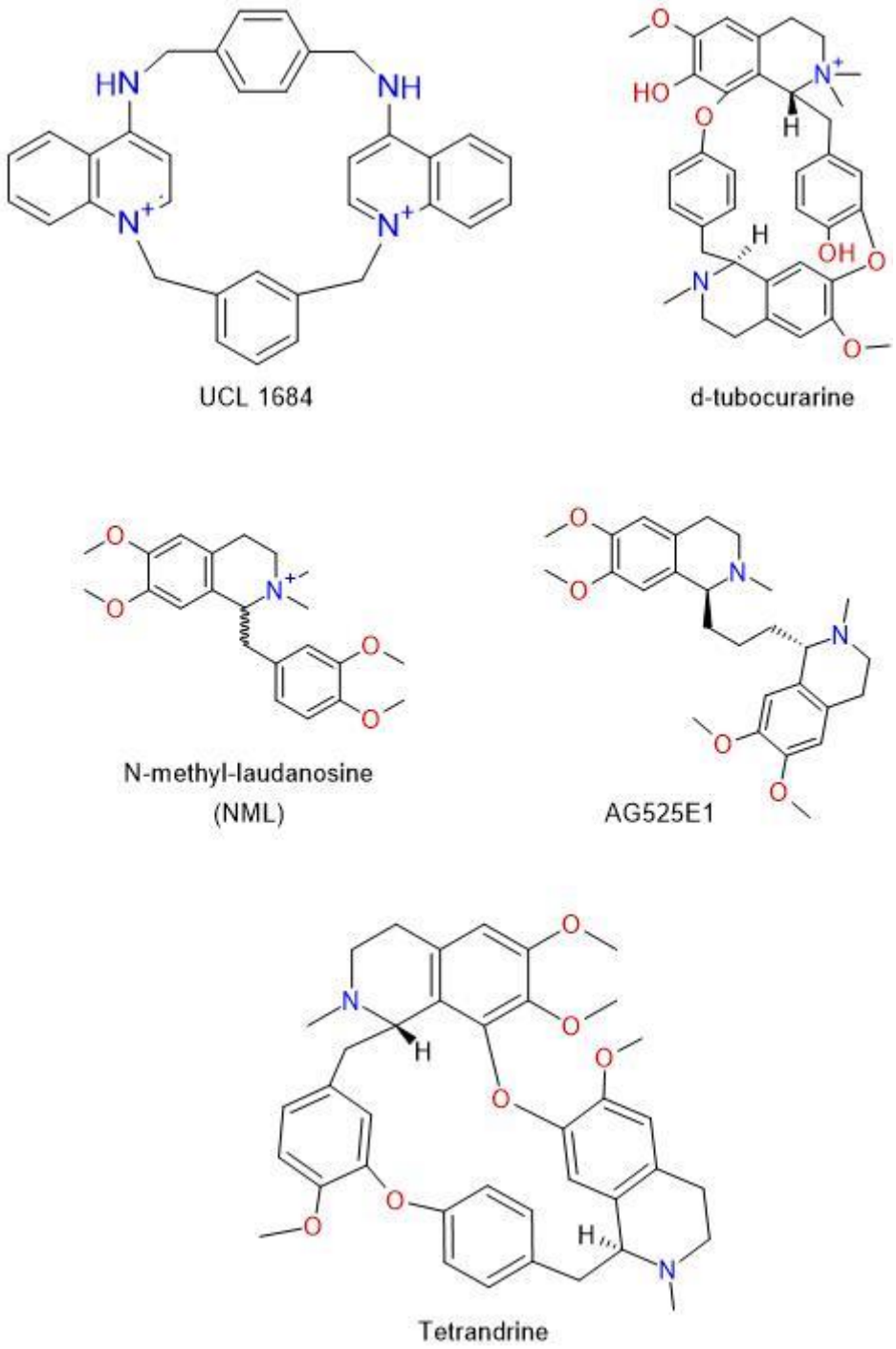

Fig. 2 : Chemical structures of previously investigated THIQ derived SK blockers.

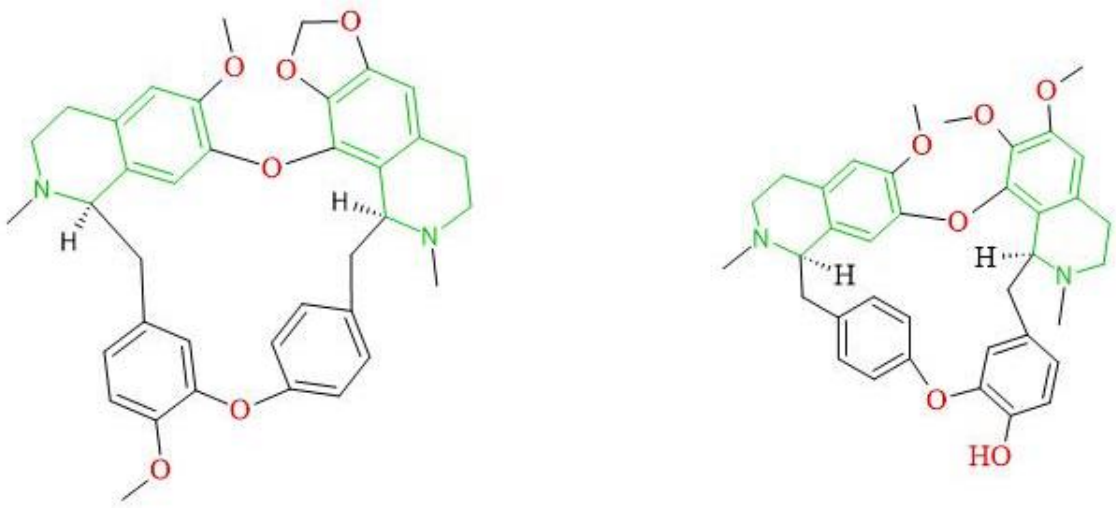

Fig. 3 : Chemical structures the bis-THIQ alkaloids Cepharanthine and Berbamine. The THIQ moieties are highlighted in green in both compounds.

The two alkaloid drugs were assessed in our radioactive binding procedure using two different types of preparations: one containing SK2 channels, the other SK3 channels. The tracer ligand used in this procedure is the radiolabeled ^{125}I-apamin.

Indeed, the majority of diseases and conditions involving the SK family are tied to one of these two subtypes. Therefore the ability of a ligand to bind to each of them separately is an important information regarding their usability in therapeutics. Furthermore, the subtype selectivity, or subtype preference, represented by the ratio between the SK2 and SK3 affinities, can only be obtained this way.

The alkaloids were evaluated with each target over 3 to 5 assays. (Fig. 4)

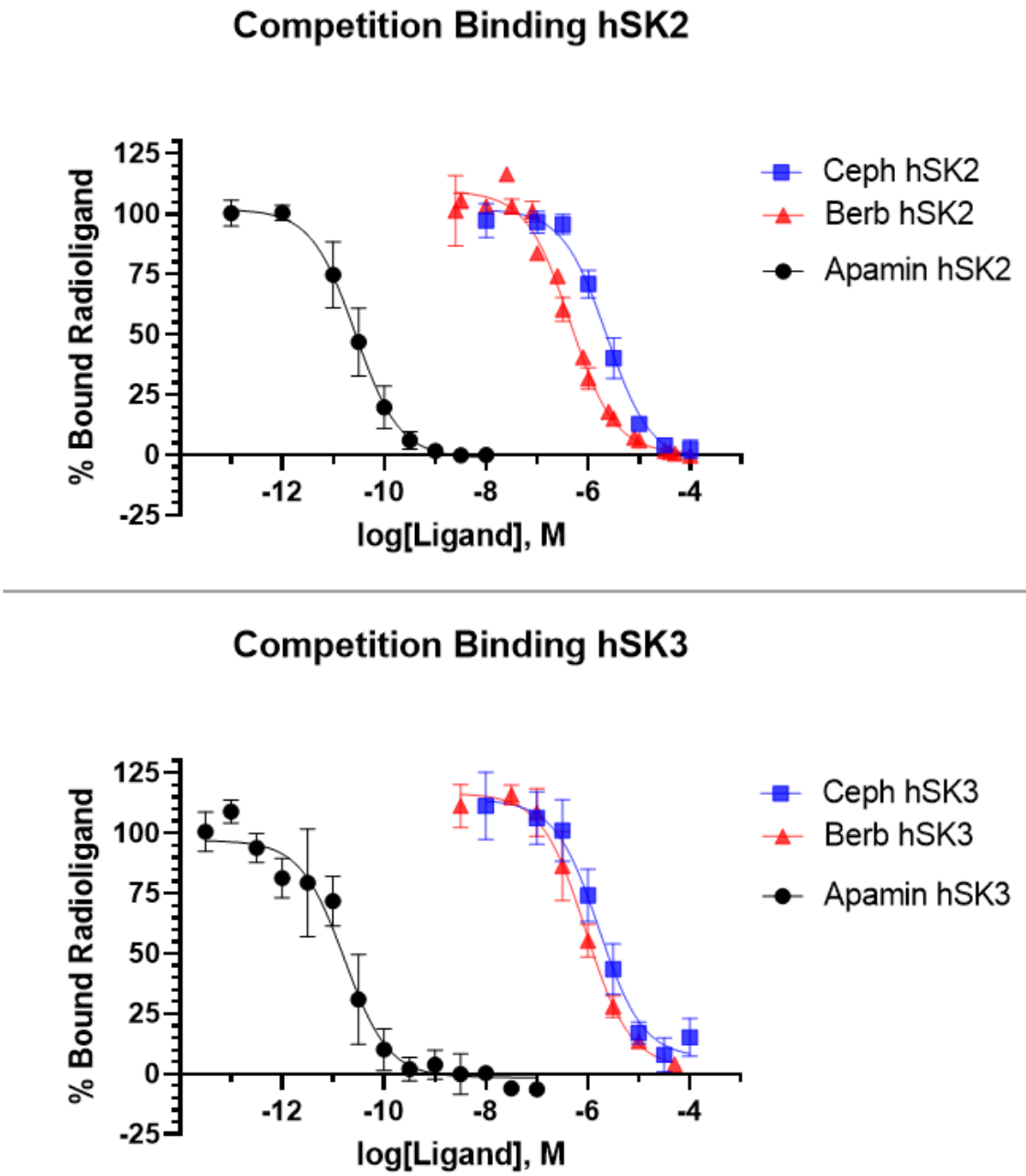

Fig. 4 : Binding competition curves obtained for the alkaloids on human SK2 channels (top), and on human SK3 channels (bottom) (n=3 to 5, confer Table 1). Results are presented in comparison to apamin obtained in the same procedure (n = 4). The displacement of ^{125}I-apamin, the radiolabeled ligand, is measured in relation to competitor concentration (Logarithmic). Curve points are means of the values from all experiments, standard deviation is represented by error bars.

The different binding curves of the competitors (Fig. 4) each yield an IC_{50} value for the affinity for the apamin binding site. That value was then transformed into K_i according to the Cheng-Prusoff equation (30) using recently obtained K_D values of the ^{125}I-apamin for the corresponding targets (i.e. 5,009 pM for hSK2 and 6,576 pM for hSK3). The mean affinity constant obtained for each competitor is presented in Table 1. At this point, some observations can be made. First, the tested compounds had significant affinity for both subtypes of SK channels. Cepharanthine and berbamine present affinities that are comparable to that of AG525E1 and its isomers, as their Ki values on SK2 and SK3 are respectively 1,318 ±0,281 µM and 1,091 ±0,191 µM for cepharanthine and 0,284 ±0,066 µM and 0,679 ±0,323 µM for berbamine (Table 1).

Furthermore, the assessed compounds of this project are bound to both SK targets with no apparent selectivity for either of the SK subtypes. Indeed, it is agreed upon that a compound can be considered selective of a given target when it displays 100 fold greater affinity for it than for any other. In this case, none of the molecules have a ratio above 2 when comparing affinity for hSK2 and hSK3. These compounds can therefore be considered non-subtype-specific ligands of the SK family (mean affinities are statistically equivalent for both subtypes, $p<0.05$).

Finally, additional testing showed that the reinforcement of the positive charges on both nitrogens, obtained by N-methylation yielding quaternary analogues, resulted in an increased affinity for SK targets (DATA not shown). This is in accordance with the pharmacophore (16-17) and with previous investigations of related molecules such as tetrandrine and its stronger *N*-methylated analogue, and the AG525 isomers which were also *N*-methylated yielding stronger derivatives (23). Such quaternary compounds are however less interesting in therapeutic contexts as their biological availability is strongly reduced due to cell-membrane impermeability, especially when considering central nervous system distribution.

Table 1: Affinities of bis-THIQ compounds for SK2 and SK3 channels. Values are presented as mean ± SD. For original data, the number of experiments realized to obtain each value is reported between brackets.

° published K_i affinities (21-23), previous SK2 affinities were evaluated on the rat "rSK2" subtype

Ligand	hSK2 Ki (µM) (number of experiments)	hSK3 Ki (µM) (number of experiments)	SK2-Selectivity factor (subtype preference)
cepharanthine	1,318 ±0,281 (3)	1,091 ±0,191 (4)	0.83
berbamine	0,284 ±0,066 (5)	0,679 ±0,323 (4)	2.17
AG525 E1°	0,277 ±0,040	0,370 ±0,094	1,34
AG525 E2°	1,256 ±0,170	1,625 ±0,238	1,29
AG525 meso°	1,685 ±0,149	1,791 ±0,147	1,06
tetrandrine°	0,306 ±0,050	0,461 ±0,042	1,51

This newfound SK interaction is of major importance when considering these alkaloids' pharmacological interest. Indeed, while both have been used commonly for quite some time, their complex mode of action is still unclear today (31,32). Considering SK channels as one of the potential targets of these multi-indication drugs may help to explain some of their therapeutic effects, several interesting examples are discussed below.

Indeed, when used in therapy, cepharanthine doses usually range from 10 mg per day to sometimes 100 mg per day. Weight-dependent doses used in clinical studies can even go up to 6 mg / kg per day, which amounts to a few hundred milligrams per dose (33). It has also been studied in several contexts such as the treatment of leukopenia, cancer management therapies and as an antiviral. Its reported active concentrations are mostly situated between 1 and 10 µM depending on the application.

Similarly, berbamine has been used at 150 mg / day for the treatment of patients with cancer (34). It also has reported effective concentrations *in vitro*, ranging from 1 to 20µM, for several studies concerning cytotoxic effects, antiproliferative effects and cell migration inhibition effects, for example.

It is now remarkable that at usual therapeutic concentrations, significant SK interactions would occur for both alkaloids based on the affinities determined in this study. Furthermore, for a good number of pathological pathways in which cepharanthine and berbamine are studied, SK channels have also been proven to take part.

In the context of cancer pathologies, both alkaloids have interesting properties. Cepharanthine is notably able to provoke apoptosis in Jurkat and K562 human leukemia cell lines (35), and to inhibit lung cancer metastases (36,37). Berbamine itself can inhibit the growth of the NB4 leukemia cell line while inducing their apoptosis as well (38). It also showed a dose dependent effect on invasion and metastasis in liver cancer cells SMMC-7721 (39). SK channels are well known to play a part in the development of several types of cancers (8-10), especially under hypoxia (SK2) (11). Hence, their modulation could have an effect on the survivability and growth of cancerous cell lines. In addition, the SK3 channel subtype is involved in the metastatic process of several types of cancers, including breast cancer (7) and melanoma (13). Therefore, SK3 channel blockers are considered as potential anti-metastatic agents.

One specific type of cancer, hepatocellular cholangiocarcinoma, has shown to be particularly interesting in this case as it is responsive to treatment by these alkaloids. Cepharanthine has been shown to induce mitophagy in these cells (40), and inhibited their growth, proliferation and viability (41). According to investigations of this pathology, SK channels of the SK3 subtype would be responsible for the metastatic events in hepatocellular cholangiocarcinoma and might be one of the driver genes involved (42). SK modulation being a valuable therapeutic strategy is therefore a strong possibility in the treatment of this specific condition.

Other types of liver injuries respond well to the two alkaloids. Berbamine reduced hepatic inflammatory phenomena in mice with ethanol-induced liver injuries (43). Cepharanthine,

studied in association with limb ischemia-reperfusion on rats, was able to improve liver function while reducing nitric oxide, malondialdehyde and macrophage inflammatory protein levels. (44)

Once again, SK channels are interestingly correlated to this topic, as a few studies showed that both subtypes can be expressed in the liver and that the modulation of the SK2 subtype is beneficial against fibrotic liver injuries. (45,46)

In another system entirely, SK2 channels are also investigated as potential targets for the treatment of Alzheimer's Disease and other pathologies reducing cognitive functions. In the central nervous system, SK channels are very well represented, with a predominance of SK3 subtype in basal nuclei, while the main subtype in the cortical and hippocampal regions is SK2. (47) Neuron populations treated with apamin show improved synaptic plasticity as well as better hippocampal dependent learning. Moreover, the *in vivo* investigations in mice focusing on learning and memory processes showed that the blockade of SK channels by apamin improved all those functions (48-50). A similar effect has been shown for mice treated daily for 40 days with 280 mg / Kg of berbamine. The *in vivo* study involved a mouse model of Alzheimer's Disease, and highlighted improved memory abilities as well as positioning learning. The treatment also alleviated the hippocampal tissue damage typically found in this animal model (51).

Cepharanthine also displayed cognitive enhancing properties, especially when associated with dexmedetomidine (52). Rats of a senile dementia model receiving cepharanthine alone or in combination scored higher in assays of cognitive and neurological functions.

In conclusion, we were able to put forward an interesting and potent affinity of berbamine and cepharanthine for both of the main SK channel subtypes. Given our reference blocker, the competition binding assay also confirms that both drugs bind the targets at the apamin sensitive site, which leads to a strong hypothesis that they can be efficient current blockers of the channels. This biological interaction occurs at concentrations that fit with usual therapeutic and preclinical use of the drugs, thus possibly describing a new mode of action for these alkaloids. Their ability to modulate SK currents in live cells must now be assessed. From these findings, we also conclude that bis-THIQ alkaloids represent an interesting source of potent ligands of SK channels. Other compounds sharing these structural characteristics will therefore be assessed as ligands of the SK family, potentially *in silico*, but also in affinity and electrophysiological assays. If other known drugs are found to bind similarly to the SK family, their known mode of action could also be complemented, or even reinterpreted.

The discovery of such SK affinities for known drugs can also be utilized to study the involvement of the SK family in different physio-pathological settings.

Supporting Information:

- Commercial sources for products
- Detailed experimental procedures for cell culture and transfection, biological material preparation and affinity binding measurements

Author Contributions:

R.V. experimental design, realization of binding experiments, analysis and interpretation of data, preparation of biological material, draft writing and editing; H.T. conception, realization of binding experiments, draft writing; M.D. realization of binding experiments, analysis of data, draft revisions; J.F.L. conception, realization of binding experiments, draft revisions, funding acquisition, supervision.

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Conflict of interest:

The authors declare no competing financial interest.

Abbreviations:

The abbreviations used in this text are: SK [channel], small conductance calcium-activated potassium [channel]; THIQ, tetrahydroisoquinoline

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