Application of BACE1 immobilized enzyme reactor for the characterization of multifunctional alkaloids from *Corydalis cava* **(Fumariaceae) as Alzheimer's disease** targets

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Abstract

In our ongoing study focused on *Corydalis cava* (Fumariaceae), used in folk medicine in the treatment of memory dysfunctions, we have investigated fifteen previously isolated alkaloids for their potential multifunctional activity on Alzheimer's disease (AD) targets. Determination of ß-site amyloid precursor protein cleaving enzyme 1 (BACE1) inhibition was carried out using a BACE1-Immobilized Enzyme Reactor (IMER) by validating the assay with a multi-well plate format Fluorescence Resonance Energy Transfer (FRET) assay. Seven alkaloids out of fifteen were found to be active, with (–)-corycavamine (3) and (+)-corynoline (5) demonstrating the highest BACE1 inhibition activity, in the micromolar range, in a concentration dependent manner. BACE1-IMER was found to be a valid device for the fast screening of inhibitors and the determination of their potency. In a permeation assay (PAMPA) for the prediction of blood-brain barrier (BBB) penetration, the most active compounds, (–)-corycavamine (3) and (+)-corynoline (5), were found to be able to cross the BBB. Not all compounds showed activity against glycogen synthase kinase-3β (GSK-3β) and casein kinase-1δ (CK-1δ). On the basis of the reported results, we found that some *Corydalis cava* alkaloids have multifunctional activity against AD targets (prolyl oligopeptidase, cholinesterases and BACE1). Moreover, we tried to elucidate the treatment effectivity (rational use) of its extract in memory dysfunction in folk medicine.

Keywords: BACE1 inhibitors, immobilized enzyme reactor, *Corydalis cava* alkaloids, PAMPA assay

Chemical compounds studied in this article:

Canadine (PubChem CID: 443422); Corycavamine (PubChem CID: 90478581); Corynoline (PubChem CID: 177014); Corycavidine (PubChem CID: 12304033); Canadaline (PubChem CID: 321459); Allocryptopine (PubChem CID: 98570); Isocorypalmine (PubChem CID: 10220); Corypalmine (PubChem CID: 12304090); Scoulerine (PubChem CID: 22955)

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative progressive disorder and the most common form of dementia amongst the elderly. AD is characterized by pathological hallmarks: selective cholinergic neuronal loss, intracellular neurofibrillary tangles (NFTs) composed of abnormally intracellular phosphorylated τ-protein, and extracellular senile plaques (SPs) [1]. SPs are deposits of β-amyloid peptide (Aβ) which originate in abnormal processing of amyloid precursor protein (APP) [2]. Aβ production from APP depends on activities of the enzymes α-secretase, β-secretase (BACE1) and γ-secretase. However, during the nonamyloidogenic pathway α-secretase cleaves APP at a site within the Aβ domain, thus precluding Aβ formation. Within the amyloidogenic pathway, the proteolytic sequential action of BACE1 and γ-secretase lead to the production of neurotoxic Aβ peptides from APP [3,4]. Thus, inhibitors of BACE1 and γ-secretase represent possible therapeutic hits for nonsymptomatic AD treatment. Although γ-secretase inhibitors triggered toxic effects in human trials, in the case of γ-secretase knockout mice the effect was lethal at their embryonic stage; this enzyme has various physiological functions and it is essential for normal development [5]. On the other hand, BACE1 inhibition decreased Aβ production, and BACE1 knockout in mice terminated Aβ40 and Aβ42 production without any severe dysfunction [6]. Therefore, BACE1 inhibition has become a promising approach for AD treatment. One of the neuropathological characteristics of AD is the presence of neurofibrillary tangles (NFTs) consisting of paired helical filaments, with the main component being hyperphosphorylated τ-protein [7]. Phosphorylation of τ-proteins is primarily dependent on GSK-3β and cyclin-dependent kinase 5 (CDK5) [8]. GSK-3β represents the isoform of GSK-3. This enzyme is involved in glycogen synthesis, but more recently, GSK-3 has been found to play an important role in the central nervous system (CNS) [9,10]. When the enzyme is dysregulated, GSK-3 proves to be a pathogenic molecule in tissues – in the CNS the enzyme is associated with neurodegenerative disorders, stroke and mood disorders [11]. Specifically in AD patient brains, abnormally high GSK-3 activity is linked with τ-hyperphosphorylation, as mentioned above, but also with memory impairment, increased Aβ production and local plaque-associated microglial-mediated inflammatory responses [12]. Moreover, casein kinase-1δ (CK-1δ) belongs to the kinases responsible for τ-hyperphosphorylation as well. The CK-1 activity is stimulated by Aβ and the enzyme is involved in the regulation of Aβ production in neurons [13]. Thus, CK-1δ and GSK-3β inhibitors offer another valuable approach toward AD therapies.

Given the complexity and the interconnected pathological pathways of the disease, lately multi-target drug approaches have been carried out to provide a more effective treatment for AD. A multi-target directed ligand is able to modulate simultaneously more than one neurogenerative pathway, for example amyloid formation and cognitive functions, when more than one activity is shown by the same molecule. Another potential therapeutic approach consists of combining active molecules, each one hitting one specific target. These strategies have explored inhibitors acting on different sites of the acetylcholinesterase enzyme, producing at least two different activities for multi-target drugs acting on different AD targets [14–19].

In previous studies, several species of *Corydalis* have been reported to be used in folk medicine for treating memory dysfunction [20,21]. Tubers of *C. cava* are a rich source of isoquinoline alkaloids of different structural types [22–26]. In our previous study focused on tertiary alkaloids from *Corydalis cava* (L.) Schweigg. et Koerte (Fumariaceae), some potent inhibitors of human cholinesterases were found [26]. In the current study we decided to elucidate if the previously isolated compounds (+)-canadine (1), (+)-corydaline (2), (–)-corycavamine (3), (+)-tetrahydropalmatine (4), (+)-corynoline (5), (±)-corycavidine (6), (+)-canadaline (7), allocryptopine (8), (+)-corydine (9), (+)-bulbocapnine (10), (–)-isocorypalmine (11), (+)-corypalmine (12), (–)-scoulerine (13), (–)-sinoacutine (14), and (+)-*N*-methyllaurotetanine (15) trigger inhibition effects which could be valuable or useful for the potential prevention of nonsymptomatic AD. Therefore, their potential inhibition effects on BACE1, GSK-3β and CK-1δ were evaluated. BACE1 inhibition by natural products has become interesting for finding potential drugs with anti-AD effects. Several potent BACE1 inhibitors have been discovered, mainly among nonalkaloidal compounds, but recent studies have confirmed BACE1 inhibition activity of alkaloids as well [3, 27–30]. Similarly, GSK-3β and CK-1δ have become promising targets for AD treatment [31,32]. A review by Martinez *et al.* reported that several potent inhibitors of GSK-3β have been discovered from marine organisms [31]. Moreover, compounds derived from natural sources are often endowed with unique and original scaffolds, which are very promising in terms of new chemical structures to be explored in relation to multi-target activities [14].

The most common methods used for determination of BACE1 inhibition activities of compounds involve fluorimetric assays in multi-well plates based on the FRET principle. FRET assays are sensitive and homogenous, but have several basic drawbacks such as high enzyme consumption, long analysis times, variability in the different sources and concentration of enzymes, and impossibility of re-usability of the used enzyme, as well as

complex robotic liquid handling. Furthermore, many compounds can produce false positive or negative results due to their limitations with solubility under FRET assay conditions, which lead to production of aggregates or due to interference with substrate hydrolysis products, because some compounds can possess fluorescent aromatic and/or heterocyclic moieties in their structures [33]. On the other hand, the use of immobilized enzymatic systems has proved a valid alternative to solution enzymatic assays, showing the advantages of speed, automation and reusability of the immobilized enzyme reactor [33–35].

Finally, if compounds have to be considered as drug candidates for AD treatment, it is necessary to determine their ability to cross the blood–brain barrier (BBB), which is a necessary feature of all potential substances for the treatment of CNS–related disorders. The BBB is a protective system that prevents substances and molecules from the blood from entering into the brain. Molecules which are soluble in lipids are able to penetrate relatively easily through the BBB via lipid cell membranes. On the other hand, hydrophilic molecules cross the barrier by use of specialized carrier–mediated transport mechanisms only [36]. The parallel artificial membrane permeability assay (PAMPA) is a model assay for determination of penetration via the BBB. The PAMPA is a high throughput method developed for prediction of passive permeability through biological membranes, employing a brain lipid porcine membrane [37].

Based on these all considerations, we aimed to disclose any multi–target activity of the alkaloids of *C. cava* by determination of their potential inhibition effects on BACE1, GSK–3β and CK–1δ, and obtained results from this study to combine together with their previously determined anti–AD activities (inhibition of prolyl oligopeptidase and human cholinesterases). We used an on–line HPLC system for BACE1 characterization of *C. cava* alkaloids by validation through FRET assay. The most active alkaloids in this study were tested in the permeation assay for the prediction of BBB passive penetration ability to explicate the putative treatment effectivity (rational use) of its extract in memory dysfunction in folk medicine.

2. Results and discussion

In order to validate a HPLC system, FRET assays using a human recombinant BACE1 (hrBACE1) in multi–wells in "solution studies" were also carried out. Compounds 1, 9, 10, and 15 gave raise to fluorescent interference with the substrate hydrolysis product of M–2420. For these compounds the substrate M–2420 could not be used, and, therefore, they were

screened with the fluorogenic Panvera peptide (Rhodamine–Glu–Val–Asn–Leu–Asp–Ala–Glu–Phe–Lys–quencher) **at higher excitation and emission wavelengths (λ_{exc} = 544 nm, λ_{em}** = 590 nm). The results of inhibition studies are reported in Table 1. Firstly, all compounds were screened at 5 μM for their ability to inhibit hrBACE1 by both the on–line HPLC assay and in solution FRET assay.

The hrBACE1–IMER values of their activity were found to be in agreement with those obtained by FRET assay. Compounds 3, 5, 6, 7, 8, 11, and 13 were found to be active as BACE1 inhibitors at a concentration of 5μM. These compounds did not interfere with the M–2420 substrate, therefore FRET and IMER assays (using the M–2420) were performed and their IC_{50} values determined.

Compounds 3 and 5 (Figure 1) demonstrated BACE1 inhibition activity in a concentration dependent manner in both assays and their IC_{50} values were determined. 3: IC_{50} FRET = 41.16 ± 7.82 μM; IC_{50} hrBACE1–IMER = 1690 ± 545 μM and 5: IC_{50} FRET = 33.59 ± 0.23 μM; IC_{50} hrBACE1–IMER = 89.07 ± 15.08 μM. Our results for BACE1 inhibition activity of the above mentioned alkaloids were in the micromolar range, which is in line with literature data obtained for structurally similar compounds [29].

Table 1

BACE1 inhibition activity of *Corydalis cava* alkaloids in FRET and IMER assays

Compound	FRET[a]		IMER[a]	
	% inhibition (5 μM)	IC_{50} (μM)	% inhibition (5 μM)	IC_{50} (μM)
1	n.i.[b]		n.t.[c]	
2	n.i.		n.i.	
3	34.40 ± 6.28	41.16 ± 7.82	17.70 ± 0.10	1690 ± 545.0
4	n.i.		n.i.	
5	21.91 ± 4.00	33.59 ± 0.23	12.05 ± 0.85	89.07 ± 15.08
6	26.87 ± 0.05		15.86 ±2.64	
7	41.02 ± 5.06		18.07 ± 0.11	
8	26.18 ± 2.35		16.86 ± 4.35	
9	n.i.[b]		n.t.[c]	
10	n.i.[b]		n.t.[c]	
11	28.68 ± 1.71		17.20 ±5.82	
12	n.i.		n.i.	
13	24.34 ± 0.36		19.02 ±1.59	
14	n.i.		n.i.	
15	n.i.[b]		n.t.[c]	
Inhibitor IV[d]			0.02 ± 0.0	0.46 ± 0.06

[a] Data are the means ± SD of two independent replications. [b] FRET assay measured with the Panvera substrate; for other compounds the substrate M–2420 was used. [c] Compounds not tested due to production of fluorescent interference with M–2420. [d] BACE1 standard inhibitor. n.i. no inhibition.

In fact, Jung *et al.* found that for *Coptidis Rhizoma* alkaloids the presence of the methylenedioxy–group in the D ring is a key contributor to the BACE1 inhibition of protoberberine alkaloids. Aporphine alkaloids proved inactive with respect to BACE1

inhibition (quaternary alkaloid magnoflorine showed an IC_{50} value > 100 μM). An analogous absence of activity towards BACE1 was found for protoberberine alkaloids 1, 2, 4, and 12, with the missing methylenedioxy–group in the D ring, and aporphine alkaloids 9, 10, and 15 in our series of compounds. Interestingly, most *C. cava* alkaloids with BACE1 inhibition activity also possess the methylenedioxy–group, despite the group including different structural types of isoquinoline alkaloids (3, 6, and 8 are protopines, 5 is a benzophenanthridine alkaloid, and 7 is classified as a secoberberine alkaloid). In the case of the protopine alkaloids 3, 6, and 8 it seems that the methylenedioxy–group in 3 is responsible/necessary for BACE1 inhibition activity in a dose–dependent manner.

Even if there are differences in IC_{50} values of inhibitors tested in FRET and IMER assays the different IC_{50} values can be caused due to different conditions used in both methods (e.g. for the on–line method 30 times higher concentrated samples than that used in solution method are required, furthermore, a different contact time – an incubation time of around an hour is required for FRET assay while for on–line method the inhibitor is flushed on IMER surface at a specific flow rate for less than a minute). However, IMER methodology has been demonstrated to give the opportunity of obtain well correlated results to those obtained by in solution assays [34,35]. Moreover, the pIC_{50} values from on– and off–line studies is a key requisite in order to compare data from on–line studies with data from in solution assay [38,39]. This was well demonstrated for BACE1 known inhibitors with inhibitor activity ranging from the low nanomolar range to the high micromolar range [34]. The pIC_{50} values obtained with the IMER were compared with those obtained with the free enzyme in order to obtain the correlation graph reported in Figure 2. This graph can be also used for the extrapolation of pIC_{50} values for in solution method from those obtained with immobilized one. For compound 3 the reported data could also considered comparable. If we take into account the pIC_{50}[$-\log IC_{50}$(M)] values obtained for compound 3 by both the analytical methods it could be easier to understand that the results are well correlated. pIC_{50} values of 4.38 and 2.77 are obtained for the off– and on–line method respectively. After plotting these pIC_{50} values in the reported correlation graph (Figure 2) a linear correlation factor of 0,9739 was found. This correlation value makes the hrBACE1–IMER a valid alternative to FRET assay and a tool to assess the inhibitory potency of unknown inhibitors. We also confirmed the limit of IMER analysis that is suitable for determination of IC_{50} values of inhibitors in low concentration [35]. However, due to the lack of a dose–response effect, it was not possible to determine IC_{50} values of compounds 6, 7, 8, 11, and 13 with either method, in spite of the compounds showing 15–19% BACE1 inhibition at 5 μM in the IMER assay.

Compounds 1–15 were also evaluated for their GSK-3β and CK-1δ inhibition activities. First, the alkaloids were screened at 10 μM in both kinase assays. The alkaloids were considered as inactive (percentage inhibition was < 10 % in both cases) with respect to the known inhibitors of GSK-3β and CK-1δ {alsterpaullone (IC_{50} = 5.03 ± 0.12 nM) and IC261 (IC_{50} = 1.00 μM)} [43–44].

The BACE1 inhibitors 3 and 5 were evaluated for their ability to cross the BBB. In order to explore the capability of the tested compounds to penetrate into the brain, the PAMPA–BBB method described by Di *et al.* was used [37]. The *in vitro* permeability (Pe) of 9 commercial drugs through the lipid membrane extract, together with 3 and 5, were determined; the results are presented in Table 2. An assay validation has been previously performed comparing the reported permeability values of commercial drugs with the experimental data obtained by this method. Based on data obtained for good correlation between the experimentally described values, it was possible to classify compounds as CNS+ when they presented a permeability > 3.88×10^{-6} cm s^{-1} [45]. Therefore we can consider that alkaloids 3 and 5 are able to cross the BBB by passive permeation.

Table 2

Permeability (Pe 10^{-6} cm s^{-1}) in the PAMPA-BBB assay of nine commercial drugs (used in experimental validation) and alkaloids 3 and 5 with their predictive penetration into the CNS[a].

Compound	PAMPA-BBB		Prediction
	reported permeability[b], Pe (10^{-6} cm s^{-1})	experimental permeability[c], Pe (10^{-6} cm s^{-1})	
atenolol	0.8	0.1 ± 0.1	
caffeine	1.3	3.0 ± 0.1	
desipramine	12.0	12.7 ± 0.4	
enoxacin	0.9	0.2 ± 0.1	
hydrocortisone	1.9	0.7 ± 0.6	
piroxicam	2.5	0.2 ± 0.2	
promazine	8.8	13.3 ± 0.4	
testosterone	17.0	22.5 ± 2.5	
verapamil	16.0	18.5 ± 1.2	
3		16.3 ± 0.9	CNS+
5		11.7 ± 0.1	CNS+

[a] PBS:EtOH (70:30) was used as solvent. [b] Reference Di *et al.* [c] Data are the mean ± SD of two independent experiments.

Although some compounds in this study showed BACE1 inhibition activity only (considered inactive against GSK-3β and CK-1δ), *C. cava* alkaloids also possess multi-targeting activities for potential prevention and treatment of AD, similar to *Coptidis Rhizoma* alkaloids influencing both amyloid formation and cognitive functions by BACE1, cholinesterase inhibition and antioxidant activities [29]. In addition to the BACE1 inhibition activity mentioned above, alkaloids from *C. cava* had previously demonstrated anticholinesterase

activities towards human AChE, BuChE and prolyl oligopeptidase [26,46]. Briefly, tertiary protoberberine alkaloids of *C. cava* principally inhibited AChE, in particular 1 and 2. BuChE was inhibited mainly by the aporphine and protopine alkaloids 10 and 6, respectively [26].

In comparison, *Coptidis Rhizoma* quaternary protoberberine alkaloids exerted very potent balanced inhibition of AChE and BuChE activities with low micromolar IC_{50} values [29]. Their significant anticholinesterase activity is associated with planarity, substitutions on the molecule, positive charge and aromaticity of the nitrogen [47]. However, quaternary alkaloids might have problems with crossing the BBB [48]. Compounds 1, 5 and 12 were found to be prolyl oligopeptidase (POP) inhibitors, the activities of which were comparable with the standard, berberine, but these alkaloids are not so potent POP inhibitors as known inhibitors (e.g. *Z*-prolyl-prolinal) [46].

If we summarize the alkaloids of *C. cava* that can influence two or more targets (BACE1, cholinesterases and prolyl oligopeptidase; Table 3), the most active compounds are 1, 3, 5, 6, 7, 8, and 11, shown in Figure 1.

Table 3

Multi-target-directed ligands from *C. cava*

Compound	BACE1 IC_{50} (μM)		Human cholinesterases [26] IC_{50} (μM)		POP IC_{50} (μM) [46]
	FRET assay	IMER	AChE	BChE	
1	n.a[a].	n.t[b]	12.4 ± 0.9	483 ± 11.5	152.0 ± 12.5
3	41.16 ± 7.82	1690 ± 545.0	428.0 ± 8.4	218.0 ± 5.1	n.a.
5	33.59 ± 0.23	89.07 ± 15.08	n.a.	n.a	289.1 ± 47.0
6	26.87 ± 0.05[c]	15.86 ±2.64[c]	223.0 ± 7.8	46.2 ± 2.4	n.a.
7	41.02 ± 5.06[c]	18.07 ± 0.11[c]	20.1 ± 1.1	85.2 ± 2.2	n.a.
8	26.18 ± 2.35[c]	16.86 ± 4.35[c]	250.0 ± 2.5	530 ± 28.2	n.a.
11	28.68 ± 1.71[c]	17.20 ±5.82[c]	196.0 ± 4.2	n.a.	n.a.
galanthamine[d]			6.9 ± 0.3	156.0 ± 6.9	
berberine[e]					142.0 ± 21.5
Z-prolyl-prolinal[e]					2.75×10^{-3}
Inhibitor IV[f]			0.02 ± 0.0	0.46 ± 0.06	

[a] not active. [b] not tested. [c] % inhibition at 5 μM.[d] AChE, BChE standard. [e] Prolyl oligopeptidase (POP) standard. [f] BACE1 standard. Results of BACE1 are the means ± SD of two independent replications, whereas results of POP, AChE, and BChE inhibition are the means ± SD of three independent replications.

The alkaloid 1 did not inhibit BACE1, but demonstrated an interesting inhibition of AChE and POP. Compound 3 inhibited BACE1 with an IC_{50} value of 41.16 ± 7.82 μM (FRET assay) and it triggered moderate inhibition of AChE and BuChE (IC_{50} = 428.0 ± 8.7 μM and IC_{50} = 218.0 ± 5.1 μM, respectively), whereas 6, inhibiting BACE1 at 5 μM by 26.87 ± 0.05 % (FRET assay), demonstrated better inhibiton of BuChE (IC_{50} = 46.2 ± 2.4 μM) than of AChE (IC_{50} = 223.0 ± 7.8 μM). 5 inhibited both BACE1 (IC_{50} = 33.59 ± 0.23 μM; FRET assay) and POP (IC_{50} = 289.1 ± 47.0). 7 exerted an interesting balanced inhibition of AChE

and BuChE (IC_{50} = 20.1 ± 1.1 μM and 85.2 ± 2.2 μM, respectively) with BACE1 inhibition activity of 41.02 ± 5.06 % at 5 μM. This alkaloid may possibly gain a relevant role in the discovery of more potent multi-targeting compounds with BACE1, AChE and BuChE inhibition activity. Compounds 8, and 11 influenced both BACE1 (% inhibition at 5 μM was 26–28 %) and AChE (IC_{50} = 250.0 ± 2.5 μM and 196.0 ± 4.2 μM, respectively).

As was mentioned above, several species of *Corydalis* have been reported to be used in folk medicine for treating memory dysfunction [20,21]. To elucidate the treatment effectivity of its extract in memory dysfunction in folk medicine from the complex point of view, it is necessary to know not only neuroprotective activity of the single compounds, their penetration across the BBB but also their quantitative content in the plant material, eventuelly their adverse effects. Therefore, we decided to determine the content of alkaloids in the crude extract of *C. cava* tubers by a HPLC-UV analysis (see Suppl. material.). The tubers contain about 4–6 % alkaloids of different structural types [49]. Compound 4 and 10 represent major alkaloids in *C. cava* tubers, obtained data from the quantitative HPLC analysis correspond to those data in literature [22,49]. We determined the content of compounds 4 and 10 in the air-dried plant material as 1.25 ± 0.12 % and 2.81 ± 0.27 %, respectively. Neuroprotective activity of 4 and 10 was found to be low or mild in this study and as it was published by us previously [26,46]. Interestingly, Adsersen *et al.* published that compounds 9 and 10 exerted cholinesterase inhibitory activity against nonhuman cholinesterases (10: $IC_{50\ AChE}$ = 40 ± 2 μM and $IC_{50\ BChE}$ = 83 ± 2 μM) [50]. In our previous study the alkaloid 9 showed mild anti-cholinesterase activity on human AChE ($IC_{50\ AChE}$ = 208.0 ± 6.8 μM) [26]. The HPLC determination showed that 9 was the third major alkaloid in the extract (1.02 ± 0.10 %). Based on the HPLC analysis, other isolated *C. cava* alkaloids represent minor alkaloids, the content for each compound was < 0.57 % in the dried material. It follows that their contribution to neuroprotective activity of *C. cava* tuber extracts is mild. Therefore, the treatment effectivity by *C. cava* extracts in memory dysfunction in folk medicine is not corroborated by alkaloids inhibition potency due to their quantitative content.

3. Conclusions

Fifteen alkaloids previously isolated from *Corydalis cava* (Fumariaceae) were evaluated for their multi-target activity on BACE1, GSK-3β and CK-1δ. BACE1 inhibition activity was measured using a HPLC (hrBACE-IMER) assay and in multi-well plate format FRET assays, validating the immobilized enzyme system. Alkaloids 3 and 5 demonstrated interesting

BACE1 inhibition activity both in the FRET and HPLC assays. The use of different assays for the evaluation of BACE1 inhibition provided a suitable approach to confirm results obtained by this method, at the same time eliminating both disadvantages and limits usually experienced with the aforementioned methods (FRET and HPLC). Moreover, isolated alkaloids 3 and 5 were tested for their ability to cross the BBB. Based on PAMPA–BBB assay results we can consider that 3 and 5 are able to cross the BBB by passive permeation. Thus, compounds 3 and 5 might well be regarded as lead structures for development of more potent BACE1 inhibitors as potential drugs with anti–AD effects due to their small molecular size and their ability to penetrate into the brain. All isolated compounds were screened for GSK–3β and CK–1δ inhibition activity. Unfortunately, none of the tested alkaloids showed any activity.

This study disclosed a multi–target activity of *C. cava* alkaloids. Compounds 3, 6, 7, 8, and 11 inhibit BACE1 and they are also cholinesterase inhibitors, 1 inhibits AChE and POP, whereas 5 inhibits both BACE1 and POP. Therefore the efficacy of the use of this plant in folk medicine in treating memory dysfunctions could be explained from the point of view of BACE1, cholinesterase and POP inhibition, along with BBB predicted penetration of the most potent BACE1 inhibitors 3 and 5. Due to quantitative low content of compounds with neuroprotective activity in the plant material, the use of *C. cava* extracts in the therapy of memory dysfunction in folk medicine is not corroborated by ascertained data.

4. Material and methods

4.1. Materials and apparatus

BACE1 FRET assays were carried out using a Fluoreskan Ascent spectrofluorometer (beam diameter: 3 mm; LabSystems, Helsinki, Finland) in black, 96–microwell Corning plates (Sigma–Aldrich, Milan, Italy). Sodium acetate, sodium azide, CHAPS, HEPES, triethanolamine, ammonium chloride, DMSO and hrBACE1 were purchased from Sigma–Aldrich (Milan, Italy). Inhibitor IV was obtained from Merck (Darmstadt, Germany), Panvera peptide from Invitrogen (Milan, Italy), and the peptide substrate M–2420 from Bachem (Weil am Rhein, Germany). UV and FP spectra for interference detection with the substrate M–2420 were measured on a UV/VIS Lambda Bio 20 spectrometer (Perkin Elmer, Massachusetts, USA) and a Jasco FP–6200 spectrofluorometer (Jasco, Cremella, Italy), respectively. HrBACE1–IMER was prepared and characterized as previously reported [34]. The IMER was inserted into the HPLC system consisting of a Jasco PU–1580 solvent delivery system

connected to a Jasco auto sampler model AS–2055, as well as to a Jasco FP–20 detector system (Jasco, Cremella, Italy). Water used for the preparation of solutions and mobile phases was purified by a Milli–Rx apparatus (Millipore, Milford, MA, USA). The buffer solutions were filtered through a 0.45 µm membrane filter and degassed before use in HPLC. Stock solutions of M–2420, Panvera peptide and inhibitors were prepared in DMSO and the following dilutions obtained in the appropriate buffer.

Chemicals for GSK–3β and CK–1δ assays: HEPES, EGTA, EDTA, magnesium acetate, magnesium chloride, ATP, sodium azide, Brij–35, and casein from bovine milk, 5%, were purchased from Sigma–Aldrich (Spain), GSK–3β, CK–1δ and GS2 (Phospho–Glycogen Synthase Peptide–2) from Merck Millipore (Spain), and Kinase–Glo reagent from Promega (USA). Luminescence was measured for kinase assays on a POLARstar Optima (BMG Labtech, Offenburg, Germany) multimode reader.

Chemicals and materials for PAMPA–BBB assay: nine commercial drugs (caffeine, enoxacine, hydrocortisone, desipramine, piroxicam, testosterone, promazine and verapamile and atenolol), phosphate buffer saline solution at pH 7.4 (PBS), ethanol and dodecane were purchased from Sigma, Across Organics, Merck, Aldrich and Fluka, and the porcine polar brain lipid (PBL) from Avanti Polar Lipids (Madrid, Spain). A 96–well filtrate plate (Multiscreen® IP Sterile Plate PDVF membrane, pore size 0.45 µM) served as the donor plate, and an indented 96–well plate (Multiscreen®) as the acceptor plate, both from Millipore. Filter PDVF membrane units (diameter 30 mm, pore size 0.45 µm) from Symta (Madrid, Spain) were used to filter the samples. A 96–well plate UV reader (Thermo Scientific, Multiskan spectrum) was used for UV measurements.

4.2. Corydalis cava alkaloids

The detailed procedure of extraction and isolation of 15 alkaloids from tubers of *Corydalis cava* has been previously described [26]. Purity of isolated compounds was ≥ 95 % (by NMR and GC).

4.3. BACE1 inhibition – FRET assay with the substrate M–2420

Inhibition studies were performed according to the following procedure [34]: 5 µL of tested compound (or DMSO) were pre–incubated with 175 µL of enzyme (17.2 nM, final concentration) in 20 mM sodium acetate, pH 4.5, containing CHAPS (0.1 %, w/v) for 60 minutes at room temperature. To start the reaction, 20 µL of M–2420 (3 µM, final concentration in HEPES 10 mM, pH 7.5) was added to the well and left to react for 15 minutes at 37 °C. The fluorescent signal was read at λexc = 320 nm and λem = 405 nm. The final concentration of DMSO in the mixture was kept below 5 % so that enzyme activity was

guaranteed without significant loss. Fluorescent intensities with and without inhibitor were compared and the percent inhibition in the presence of a tested compound was calculated. The background signal was measured in control wells containing all the reagents, except hrBACE1, and subtracted. The % inhibition was calculated by the following expression: 100 – (IFi/IF0 × 100), where IF_i and IF_0 are fluorescent intensities obtained for hrBACE1 in the presence and absence of inhibitor, respectively). Inhibition curves were obtained for each compound that showed correlation between concentration and enzyme inhibition by plotting the percentage inhibition versus the logarithm of inhibitor concentration in the assay sample. The linear regression parameters were determined and IC_{50} values extrapolated (GraphPad Prism).

4.4. BACE1 inhibition hrBACE1–IMER assay

Inhibition studies were performed according to the following modified procedure [34]: The fluorescence detector was set at 320 nm and 420 nm for excitation and emission, respectively. A running buffer consisted of a mixture composed of 50 mM ammonium chloride, 25 mM triethanolamine, CHAPS (0.1 %, w/v) at pH 4.0 and DMSO in the volumetric ratio 95/5; the flow rate was set at 1.0 mL/min and the injection volume at 10 µL. Chromatographic analyses were performed at room temperature. When not in use, the hrBACE1–IMER was stored at 4 °C in sodium acetate buffer (50 mM, pH 6.0) containing 0.1 % (w/v) sodium azide as storage buffer. The final concentration of test compound in the EDA–CIM disc with immobilized hrBACE1 was 5 µM and 1.6 µM of the substrate M–2420 (normalized concentrations), respectively.The final concentrations in the IMER were calculated according to the formula: (Cinj × Vinj)/BV where C_{inj} is the injected concentration of either the test compound or the substrate, V_{inj} is the injected volume (0.01 mL) and BV is the bed volume of the hrBACE1–IMER (0.34 mL). The % inhibition was calculated by evaluating the product peak area with and without inhibitor according to the following formula: 100 – (Ai/A0 × 100) where Ai is the peak area calculated in the presence of the test inhibitor and A0 is the peak product area obtained with the substrate solution only. IC_{50} values of test compounds were determined by the IMER assay for compounds for which IC_{50} values were evaluated by FRET assay: alkaloids 3 and (+)–5. Inhibition curves were obtained for each compound by plotting the percentage inhibition versus the logarithm of inhibitor concentration in the assay sample. The linear regression parameters were determined and IC_{50} values extrapolated (GraphPad Prism).

4.5. BACE1 inhibition – FRET assay with Panvera peptide

Inhibition studies were performed according to the following procedure [35]: Stock solutions of test compounds were prepared in DMSO and then diluted in 50 mM sodium acetate buffer,

pH 4.5. Specifically, 20 µL of either test compound (5 µM, final concentration) or 20 mM sodium acetate was pre–incubated with 20 µL of enzyme (11.7 nM, final concentration) for 60 minutes. To start the reaction, 20 µL of the Panvera peptide (0.25 µM, final concentration) was added equally to each well. The mixture was incubated at 37 °C for 60 minutes. To stop the reaction, 20 µL of BACE1 stop solution (sodium acetate 2.5 M) was added to each well. Then, **the fluorescent intensities were recorded at λ_{em} = 590 nm (λ_{em}** = 545 nm). The percentage inhibition was determined in the same way as in previous BACE1 assays.

4.6. GSK–3β assay

The assay for determination of GSK–3β **inhibition was performed according to the** luminescent method of Baki *et al.* using Kinase–Glo reagent in assay buffer in 96–well plates [51]. Ten µL (10 µM) of test compound dissolved in DMSO at 1 mM concentration was diluted in advance in assay buffer to the desired concentration. Ten µL (20 ng) of enzyme was added to each well followed by 20 µL of assay buffer containing 50 µM substrate and 2 µM ATP. The final concentration of DMSO in the reaction mixture did not exceed 1 %. After 30 min incubation at 30 °C the enzymatic reaction was stopped with 40 µL of Kinase–Glo reagent. Glo–luminescence was recorded after 10 minutes. The activity is proportional to the difference of the total and consumed ATP. The inhibition activities were calculated on the basis of maximal activities measured without the presence of inhibitor.

4.7. CK–1δ assay

The assay was performed according to the slightly modified luminescent method of Baki *et al.* using Kinase–Glo reagent in assay buffer in 96–well plates [51]. The modifications involved the composition of the assay buffer, the incubation time (60 minutes) and 20 µL of assay buffer containing the substrate solution (0.1 % casein + 4 µM ATP). The assay buffer consisted of 50 mM HEPES (pH 7.5), Brij–35 (0.01 %), 10 mM $MgCl_2$, 1 mM EGTA and sodium azide (0.01 %).

4.8. Parallel artificial membrane permeability assay (PAMPA)

The assay was performed according to Di *et al.* [35]. The validation of the assay has been described previously [45].

4.8. Statistical analysis

Calculations were performed using Microsoft Excel software (Redmont, WA, USA) and GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego, CA, USA. Results of BACE1 and PAMPA assays (Table 1–3) are the means ± standard deviations of two independent replications, whereas results of POP and cholinesterase inhibition (Table 3) are the means ± standard deviations of three independent replications.

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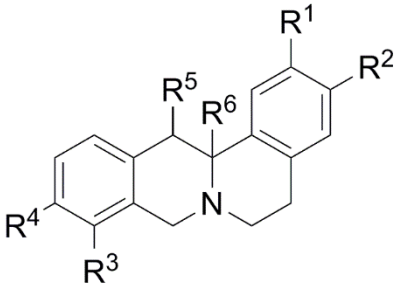
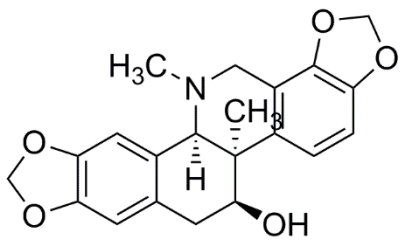
Figure

Figure 1: *Corydalis cava* alkaloids





5

1 $R^1 = R^2 = -O\text{-}CH_2\text{-}O-$, $R^3 = R^4 = -OCH_3$, $R^5 = -H$, $R^6 =$

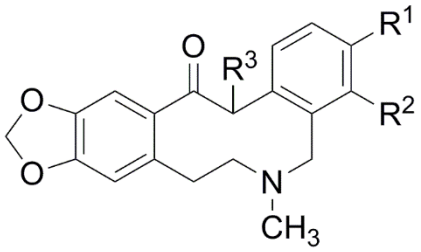
2 $R^1 = R^2 = R^3 = R^4 = -OCH_3$, $R^5 =$ CH_3, $R^6 =$

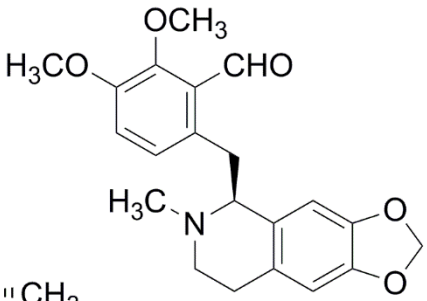
4 $R^1 = R^2 = R^3 = R^4 = -OCH_3$, $R^5 = -H$, $R^6 =$

11 $R^1 = OH$, $R^2 = R^3 = R^4 = -OCH_3$, $R^5 = -H$, $R^6 =$

12 $R^1 = R^3 = R^4 = -OCH_3$, $R^2 = OH$, $R^5 = -H$, $R^6 =$

13 $R^1 = R^3 = OH$, $R^2 = R^4 = -OCH_3$, , $R^5 = -H$, $R^6 =$



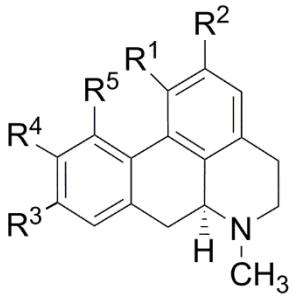


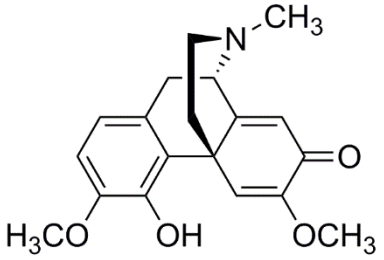
7

3 $R^1 = R^2 = -O\text{-}CH_2\text{-}O-$, $R^3 =$ CH_3

6 $R^1 = R^2 = -OCH_3$, $R^3 = -CH_3$

8 $R^1 = R^2 = -OCH_3$, $R^3 = -H$





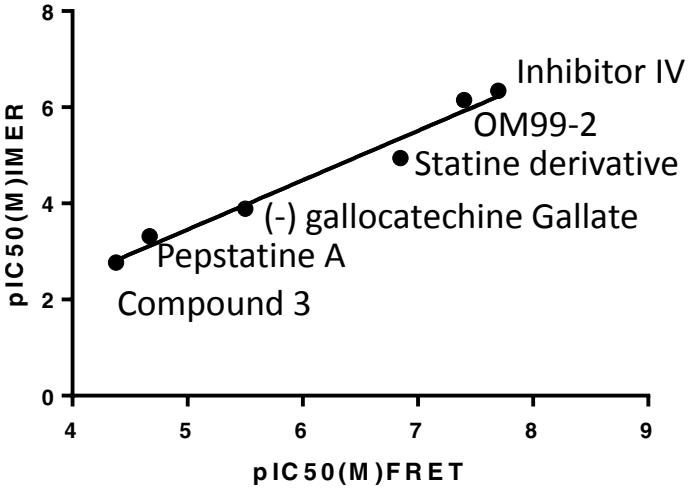
14

9 $R^1 = OH$, $R^2 = -OCH_3$, $R^3 = -H$, $R^4 = R^5 = -OCH_3$

10 $R^1 = R^2 = -O\text{-}CH_2\text{-}O-$, $R^3 = H$, $R^4 = -OCH_3$, $R^5 = OH$

15 $R^1 = R^2 = -OCH_3$, $R^3 = -OH$, $R^4 = -OCH_3$, $R^5 = -H$

Figure 2: Correlation plot for inhibitory potency (pIC50) of five known BACE1 inhibitors [40-42]. and compound 3. pIC50 values obtained by FRET assay are well correlated with those obtained by on-line methodology (r^2: 0.9739).


8
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8
9
pIC50(M)IMER
pIC50(M)FRET
Inhibitor IV
OM99-2
Statine derivative
(-) gallocatechine Gallate
Pepstatine A
Compound 3