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Original article

Chemical synthesis and biological validation of immobilized protein kinase inhibitory Leucettines

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ABSTRACT

Leucettines, a family of marine sponge-derived 2-aminoimidazolone alkaloids, are potent inhibitors of DYRKs (dual-specificity, tyrosine phosphorylation regulated kinases) and CLKs (cdc2-like kinases). They constitute promising pharmacological leads for the treatment of several diseases, including Alzheimer's disease and Down syndrome. In order to investigate the scope of potential targets of Leucettine L41, a representative member of the chemical class, we designed an affinity chromatography strategy based on agarose-immobilized leucettines. A synthesis protocol for the attachment of a polyethylene (3 or 4 units) linker to L41 was first established. The linker attachment site on L41 was selected on the basis of the co-crystal structure of L41 with several kinases. L41 was then covalently bound to agarose beads through the primary amine located at the end of the linker. Control, kinase inactive Leucettine was also immobilized, as well as free linker devoid of ligand. Extracts of several mouse tissues revealed a complex pattern of interacting proteins, some of which probably resulting from non-specific, hydrophobic binding, while others representing *bona fide* Leucettine-interacting proteins. DYRK1A and GSK-3 (glycogen synthase kinase-3) were confirmed as interacting targets by Western blotting in various mouse tissues. The Leucettine affinity chromatography resin constitutes a powerful tool to purify and identify the targets of this new promising therapeutic class of molecules.

1. Introduction

Protein phosphorylation probably constitutes one of the main intracellular regulation mechanisms. It results from the delicate balance between the activities of protein kinases and protein phosphatases. There is constantly growing evidence for links between abnormal phosphorylation and human disease, and consequently, protein kinases have now become the major class of screening targets for the pharmaceutical industry. There are currently 238 inhibitors of kinases (monoclonal antibodies and low molecular weight compounds) in clinical trials, mostly targeting tyrosine kinases and mostly targeting cancer [1].

Among the 518 human kinases, we have focused our efforts on two classes of serine/threonine kinases, DYRKs (dual-specificity, tyrosine phosphorylation regulated kinases, 5 members) and CLKs (cdc2-like kinases, 4 members). These kinases play essential physiological roles including the regulation of mRNA splicing, neuronal development, cell death, etc. They appear to be involved in various diseases including Alzheimer's disease, Down syndrome (the DYRK1A gene is located on chromosome 21, and the extra copy is associated with early signs of Alzheimer's disease in Down syndrome patients), cancer, etc, review in Refs. [2–4]. Only a limited number of pharmacological inhibitors of DYRKs and CLKs have been described and characterized [5–11]. Our screening efforts have led to the identification of a class of inhibitors derived from the marine sponge 2-aminoimidazolone alkaloid Leucettamine B [12, 13]. Starting from this natural product we have synthesized

Abbreviations: CDKs, cyclin-dependent kinases; CLKs, cdc2-like kinases; DMSO, dimethylsulfoxide; DTT, dithiothreitol; DYRKs, dual-specificity, tyrosine phosphorylation regulated kinases; EG, ethyleneglycol; GSH, glutathione; GSK-3, glycogen synthase kinase-3; GST, glutathione-S-transferase; MWI, microwave irradiation; PBS, phosphate-buffered saline; PEG, polyethylene glycol.

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over 500 derivatives which were collectively named Leucettines. The first Leucettine derivatives were described by Debdab et al. [12]. One representative and potent Leucettine, Leucettine L41, was co-crystallized with CLK3 [12], DYRK1A, DYRK2, CLK1 and PIM1 [13], allowing an excellent understanding of the mode of binding of these inhibitors to their target kinases.

We have extensively studied the selectivity of Leucettine L41 [13]. Results show that Leucettine L41 targets DYRKs and CLKs, but also interact with a few other kinases such as glycogen synthase kinase-3 (GSK-3). To identify and investigate the full scope of cellular targets of Leucettine, we have chosen to immobilize Leucettines on agarose beads and to use this affinity matrix to purify and identify the targets of Leucettines from cell lines and animal tissues. This approach has already been successfully applied to the cyclin-dependent kinase (CDKs) inhibitors purvalanol [14], roscovitine [15] and paullones [16,17]. This article describes the chemical synthesis and biological validation of immobilized Leucettines and discusses the further use of this affinity matrix in pharmacological studies.

2. Results

2.1. Chemistry

Leucettine L41 **1a** and its analogue **1b** were synthesized as previously described [12]. To identify the targets of Leucettine by affinity chromatography on agarose beads, we have developed a protocol for the synthesis of Leucettine L41 **1a** and its analogue **1b** (Fig. 1) bearing an amino-polyethylene glycol (PEG) linker on the *para*-position of the 2-phenylamino group of Leucettine. We reasoned that addition of this linker moiety at this C-4 position might enable us to immobilize the Leucettine without preventing its kinase inhibitory activity. The overall synthesis strategy for the target Leucettine derivatives **3**(**a**–**c**) with an appended PEG linker is outlined in Scheme 1.

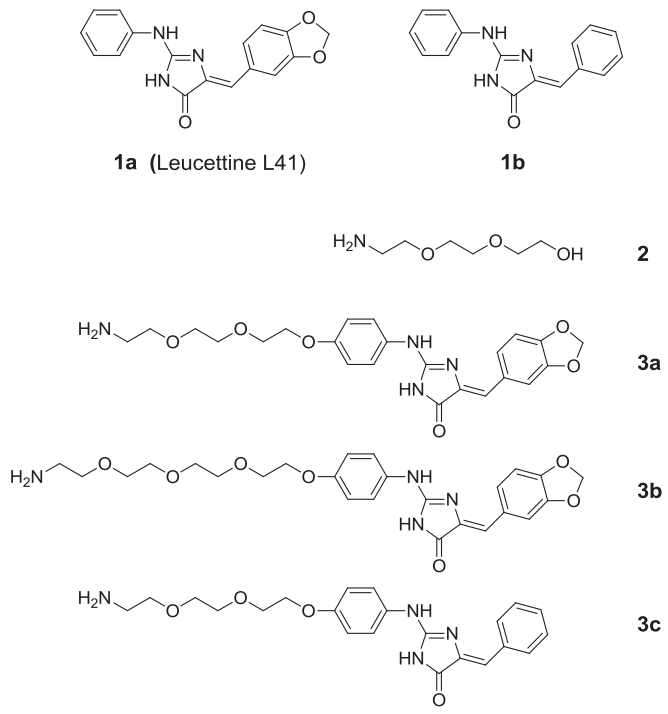
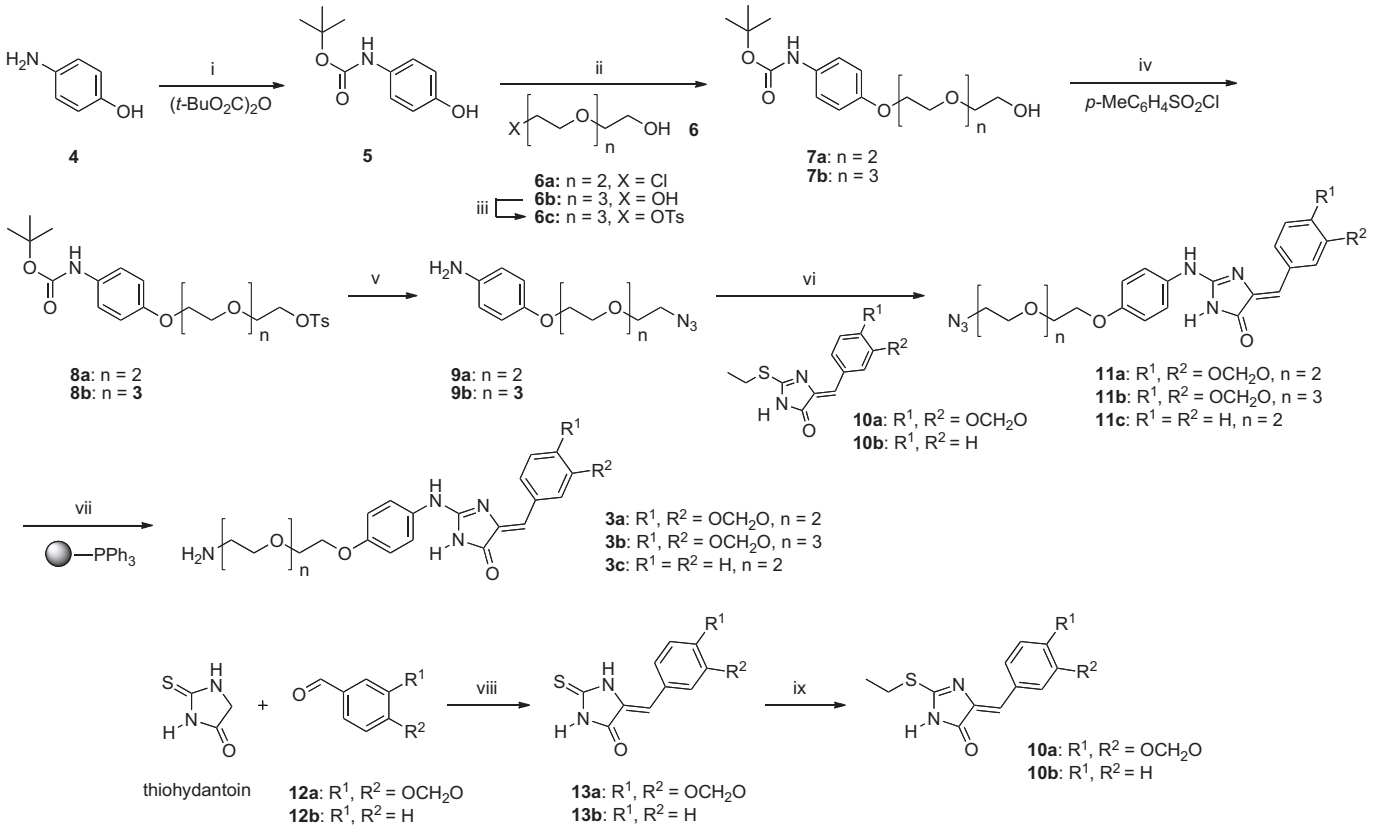


Fig. 1. Structures of compounds used. Leucettine L41 (**1a**), an inactive analogue (**1b**), free 3 EG units linker (**2**), Leucettine L41 with 3 EG units linker (**3a**), Leucettine L41 with 4 EG units linker (**3b**), compound **1b** with 3 EG units linker (**3c**).

Our first task was to perform a reliable synthesis of 4-substituted aniline **9** bearing an azido PEG fragment. Starting from commercial 4-aminophenol **4**, a regioselective protection [18] was conducted in a solution of THF by slow addition of the readily available *di-tert*-butyl dicarbonate. After 24 h, thin layer chromatography indicated consumption of the starting material and a single product **5** was obtained in quantitative yield (Table 1). Next, the *N-t*-Boc aminophenol **5** was designed to incorporate polyethylene glycol spacer arms of various lengths, respectively, from 2-[2-(2-chloroethoxy) ethoxy]ethanol **6a** for compound **7a** ($n = 2$) and 2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethoxy]ethyl-4-methylbenzenesulfonate **6c** for compound **7b** ($n = 3$). Access to the mono tosylated [19] compound **6c** could be easily realized by reaction of tetraethylene glycol **6b** with *para*-toluene sulfonyl chloride in dry THF. After reaction (2 h) at 0 °C, the desired compound **6c** was obtained in good yield (96%). In the third step, a tosyl group was introduced on **7**(**a, b**) leading to **8**(**a, b**) in moderate to good yields (47–75%) according to a modified known procedure [20]. For the fourth step, our focus was the transformation of the tosylated compounds **8**(**a, b**) into organic azide by nucleophilic substitution reaction in the presence of readily available alkali azides. The choice of this strategy was guided by the interest to use azides as a potential precursor of amines. Utilization of water as reaction media [21] in conjunction with microwave dielectric heating [22] is one of the emerging unconventional methods being recognized as potential environmentally benign alternatives. The microwave-assisted reaction of different tosylates with sodium azide was examined in an aqueous medium [23] without using a phase-transfer catalyst [24]. Water also offered a safer environmental approach to prevent the potential explosion danger of azidation in a halogenated solvent [25]. The choice of protection/deprotection strategy is highly important in organic synthesis of multifunctional molecules and a large variety of protective groups have been developed along with numerous methods for their removal. Among them, the *tert*-butoxycarbonyl (*t*-Boc) group is frequently used as a protecting group for amine and many methods for *N-t*-Boc deprotection have been reported under acidic conditions [26]. Recently, an elegant catalyst-free water-mediated deprotection of *N-t*-Boc aromatic amines has been reported [27].

In this context, our aim for this fourth step was to develop a convenient and good yielding protocol for introduction of the azide group on compound **9** associated with *N*-deprotection using water under microwave irradiation (MWI). The experiments allowed defining the optimal reaction conditions for the synthesis of **9**(**a, b**) by varying reaction temperature, power, ratio of **8** and sodium azide and composition of the solvent media for *N-t*-Boc deprotection under MWI. The optimal reaction conditions were obtained after 60 min. with a suspension of **8a** and two equivalents of sodium azide in a mixture of EtOH/H_2O (1:1) using a closed reactor to produce **9a** ($n = 2$) in 90% under MWI at 160 °C. In the same manner, the product 9**b** ($n = 3$) was also prepared in good yield (95%). Next, the second part of the synthesis concerns the construction of the 2-amino-5-arylidene imidazolin-4-one moiety bearing the azido polyethylene glycol linker. Access to compound **11** could be accomplished by sulphur/nitrogen displacement of the SEt group of an imidazolin-4-one **10** with anilines **9**(**a, b**) substituted in C-4 position by an azido PEG chain. For **10a** (R^1, $R^2 = OCH_2O$), this compound was directly synthesized according to our previous method described in literature [12]. A similar strategy was used for the synthesis of analogue **10b**. Starting from commercial thiohydantoine, the (5*Z*) 5-benzylidene-2-thioxo-imidazolin-4-one **13b** was easily obtained by Knœvenagel condensation of benzaldehyde **12b** in an AcONa/AcOH media under microwave conditions of 140 °C for 20 min using a glass tube sealed with a snap cap (**13b:** 93% yield). Then **13b** underwent S-alkylation with



Scheme 1. *Reagents and reaction conditions*: (ii) (t-$BuO_2C)_2O$, THF, 25 °C, 24 h. (ii) for **7a**: **6a**, K_2CO_3 3 equiv., NaI 0.1 eq., MeCN, 80 °C, 48 h; for **7b**: **6c**, K_2CO_3 1.5 equiv., DMF, 80 °C, 48 h. (iii) **6b**, NaOH aq., p-$MeC_6H_4SO_2Cl$, THF, 0 °C, 2 h; (iv) **7**, p-$MeC_6H_4SO_2Cl$ 2 equiv., Et_3N 3 equiv., DMAP 0.2 equiv., CH_2Cl_2, 25 °C, 24 h; (v) **8**, NaN_3 2 equiv., EtOH–H_2O (1:1), MWI, 160 °C, 60 min; (vi) **10** 1.2 equiv., MWI, 160 °C, 30 min; (vii) Ph_3P on polymer, H_2O 3 equiv., THF, 70 °C, 48 h; (viii) AcONa 1.1 equiv., AcOH, MWI, 140 °C, 20 min; (ix) EtI 1.1 equiv., K_2CO_3 0.5 equiv., Me_2NCHO, 60 °C, 24 h.

iodoethane (1.1 equiv.) in dry dimethylformamide to give the (5*Z*) 5-benzylidene-2-ethylsulfanyl-1*H*-imidazol-4-one **10b** in quantitative yield (98%) at 60 °C after a reaction time of 12 h. The structural identification of this new compound **10b** was based on the ^1H and ^{13}C assignments and was performed by extensive 1D and 2D NMR spectroscopy. **10b** was obtained solely as the *Z* isomer and no isomerization of the exocyclic double bond was observed during our synthesis. With the desired 2-ethylsulfanyl-1*H*-imidazol-4-ones **10(a,b)** in hand, we next examined the sulphur/nitrogen displacement under MWI. Reaction optimization for this reaction consisted in varying reaction temperature (from 120 to 180 °C), possible use of a solvent or not, reaction concentration (ratio **9**/**10** from 1 to 2) and reaction time (from 20 to 40 min). Sulphur/nitrogen displacement for the mixture of **9** and **10** (ratio **9a**/**10a** = 1.2) at 160 °C after 30 min led to the formation of desired product **11a** in the presence of a substantial amount of byproducts probably resulting from a cleavage of the PEG chain. We observed production of colored byproducts, possibly caused by difficulties in adequately controlling the thermal stability of the azido PEG chain on **11a** under solvent-free MWI. Analysis of the byproducts by ^1H NMR suggested cleavage of the PEG chain. Product **11a** was purified by preparative chromatography on silica gel using a step-wise gradient from 100 to 95% of CH_2Cl_2/MeOH as mobile phase (**11a**: 16% yield). In the same manner, compounds **11b** and **11c** were also prepared in very low yields (**11b**, **11c** = 6%) with another PEG length like 12 atom linker with construct **9b** or 9 atom linker with construct **9a**. For the last step, our focus was the transformation of the azido PEG imidazol-4-ones **11**(**a**–**c**) into amino imidazol-4-ones **3**(**a**–**c**) with various PEG lengths. The common methods for reduction of azides are based on hydrides [28] ($LiAlH_4$ in THF), tin(II) chloride [29] or zinc dust [30] in methanol or palladium-catalyzed hydrogenolysis [31]. This implies that a specific, tedious synthetic protocol has to be devised for each new compound. The so-called Staudinger reaction is a frequently used method [32] for smooth reduction of azides into amines using triarylphosphanes. The major drawback of this alternative method is the presence of free triphenylphosphinoxide that often contaminates the desired products. Attention was turned towards the choice of an appropriate protocol in the preparation of amino partners **3**(**a**–**c**) without

Table 1
Structure and yield of synthesized compounds.

Compound	*n*	R^1, R^2	Yield[a] (%)
5	–	–	98
6c	3	–	96
7a	2	–	39
7b	3	–	68
8a	2	–	75
8b	3	–	47
9a	2	–	90
9b	3	–	95
10b	–	H	98
11a	2	OCH_2O	18
11b	3	OCH_2O	6
11c	2	H	6
3a	2	OCH_2O	75
3b	3	OCH_2O	75
3c	2	H	76
13b	–	H	93

[a] Isolated yields.

byproducts for affinity chromatography. In this context, the use of polymer-supported triphenylphosphine in the Staudinger reaction facilitated the final purification, suppressing the formation of free triphenylphosphinoxide [33]. Finally, treatment of the azido PEG imidazolin-4-one **11a** with 2 equiv. of triphenylphosphine bound to polymer in THF during 24 h at 81 °C in the presence of water afforded the amino PEG imidazolin-4-one **3a** in 75% yield. In order to explore the question of generality, the two other amino PEG imidazolin-4-ones **3**(**b,c**) were synthesized in good yields according to the experimental procedure used for **3a**; the compounds **3b** and **3c** were obtained respectively with an isolated yield of 75 and 76%, respectively (Table 1) and in quantities suitable for affinity chromatography. The structures of the three (5*Z*) 2-(4-aminoPEG) phenylamino-5-arylidene-1*H*-imidazol-4-ones **3(a-c)** were ascertained by high-resolution mass spectrometry, proton and carbon NMR, confirming that the thermodynamically more stable *Z*-isomers were obtained.

2.2. Biology

Free Leucettine L41 **1a** and its analogue **1b**, free 3 EG units linker (**2**), 3 EG units linker tethered with Leucettine L41 **3a** or with its analogue **3c**, and 4 EG units linker tethered with Leucettine L41 **3b** (Fig. 1A), were first tested at increasing concentrations on a panel of 14 purified protein kinases. IC_{50} values were determined from the dose-response curves (Table 2). Results confirm the expected selectivity of Leucettine L41 **1a** towards DYRKs and CLKs, and GSK-3 to a lower extent. Leucettine L41 remained quite a potent inhibitor even when tethered to a 3 EG **3a** or 4 EG units linker **3b** (Table 2). The free 3 EG units linker **2** was completely inactive on the kinases. Despite the complete lack of activity of the 'kinase dead' analogue **1b**, the **1b** + linker **3c** compound showed very modest but significant effects on some kinases, including DYRK1A. These results confirm the appropriate selection of the linker attachment site on Leucettine L41 **1a**, which was based on the co-crystal structure of Leucettine L41 with various CLKs and DYRKs [12,13]. They also confirm the key Hydrogen bond between one of the oxygens of the methylene dioxy and the kinase targets (lack of or very low activity of **1b** without or with the 3 EG units linker, respectively). We thus have two active ligands (**3a, 3b**) and two inactive controls **2** and **3c**.

All four ligands were immobilized, through the linker primary amine, on agarose at a concentration of 12 μmoles/mL of gel as described in Section 3. We first ran a mouse brain extract on these affinity matrices and analyzed the bound proteins. Briefly, mouse brains were homogenized and centrifuged and equal quantities of supernatant (1 mg total protein/10 μL beads) were loaded on all four matrices. After 30 min under constant rotation at 4 °C, beads were carefully washed and bound proteins were analyzed by SDS-PAGE followed by silver staining and Western blotting using specific antibodies (Fig. 2). Results show that the absence of any ligand (with or without linker, Fig. 2, (**2**) and (**-**), respectively) provides excellent control beads which do not bind any protein from mouse brain. When Leucettines were bound to the matrix, a complex pattern was observed, whether kinase inactive (**3c**) or kinase active (**3a, 3b**) Leucettines were immobilized (Fig. 2, left panel), suggesting some non-specific, hydrophobic protein binding to the ligands. However, when specific Leucettine L41 targets were probed by Western blotting, DYRK1A and GSK-3 were found to be enriched on the kinase inhibitor beads, and much less on the control, inactive Leucettine (Fig. 2, center and right panels). However there was some modest binding of DYRK1A and GSK-3 to the inactive compound, suggesting either unspecific hydrophobic interactions or specific, but low affinity, binding, a result that fits with the modest but detectable effects of inactive Leucettine + linker on DYRK1A and GSK-3 (Table 1).

We next ran extracts of various mouse tissues on immobilized Leucettine L41 (3 EG unit linker) (Fig. 3). Silver staining revealed a different pattern of interacting proteins according to the different tissues (Fig. 3). Western blot analysis with anti-DYRK1A and anti-GSK-3 antibodies showed that these kinases are differently expressed in the different tissues and can be purified and concentrated using the Leucettine L41 matrix (Fig. 4).

Table 2
Protein kinase selectivity of Leucettine L41 (**1a**), inactive analogue (**1b**), free linker (**2**), Leucettine L41 + 3 EG linker (**3a**), Leucettine L41 + 4 EG linker (**3b**), **1b** + 3 EG linker (**3c**). Compounds were tested at various concentrations on 14 purified kinases as described in Section 3. IC_{50} values, calculated from the dose–response curves, are reported in μM. –, inactive at the highest concentration tested (10 μM); >10, inhibitory but IC_{50} >10 μM.

Kinases	1a	1b	2	3a	3b	3c
CDK1/cyclin B	>10	–(10)	–(10)	>10	>10	–(10)
CDK2/cyclin A	–(10)	–(10)	–(10)	–(10)	–(10)	–(10)
CDK5/p25	–(10)	–(10)	–(10)	–(10)	–(10)	–(10)
CDK9/cyclin T	–(10)	–(10)	–(10)	7.4	3.7	>10
CK1δ/ε	–(10)	–(10)	–(10)	>10	>10	–(10)
CLK1	0.035	–(10)	–(10)	0.075	0.061	>10
CLK2	0.32	–(10)	–(10)	0.28	0.21	–(10)
CLK3	3.0	–(10)	–(10)	0.81	0.55	–(10)
CLK4	0.031	–(10)	–(10)	0.081	0.061	>10
DYRK1A	0.021	>100	–(10)	0.05	0.028	8.5
DYRK1B	0.077	–(10)	–(10)	0.11	0.072	>10
DYRK2	0.22	–(10)	–(10)	0.15	0.13	>10
DYRK3	0.70	–(10)	–(10)	0.20	0.18	–(10)
GSK-3α/β	0.73	–(100)	–(10)	0.27	0.20	40

3. Experimental section

3.1. Chemistry

3.1.1. General

Melting points were determined on a Kofler melting point apparatus and were uncorrected. ^{1}H NMR spectra were recorded on BRUKER AC 300 P (300 MHz) spectrometer, ^{13}C NMR spectra on BRUKER AC 300 P (75 MHz) spectrometer. Chemical shifts are expressed in parts per million downfield from tetramethylsilane as an internal standard. Data are given in the following order: *d* value, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), number of protons, coupling constants *J* is given in Hertz. The mass spectra (HRMS) were taken respectively on a MS/MS ZABSpec Tof Micromass (EBE TOF geometry) at an ionizing potential of 8 eV and on a VARIAN MAT 311 at an ionizing potential of 70 eV in the "Centre Régional de Mesures Physiques de l'Ouest" (CRMPO, Rennes). Reactions under microwave irradiations were realized in the Explorer® 24 CEM microwave reactor (CEM France) and also in the Anton Paar Monowave 300® microwave reactor (Anton Paar France) using borosilicate glass vials of 10 or 30 mL equipped with snap caps (at the end of the irradiation, cooling reaction was realized by compressed air). The microwave instrument consists of a continuous focused microwave power output from 0 to 300 W for the Explorer® 24 CEM apparatus and from 0 to 800 W for the Anton Paar Monowave 300® apparatus. All the experiments were performed using stirring option. The target temperature was reached with a ramp of 3 min and the chosen microwave power maintained constant to hold the mixture at this temperature. The reaction temperature is monitored using calibrated infrared sensor and the reaction time included the ramp period. The microwave irradiation parameters (power and temperature) were monitored by the ChemDriver software package for the Explorer® 24 CEM apparatus and by the Monowave software package for the Anton Paar Monowave 300® reactor. Preparative chromatography was realized on a Combi Flash R_f 200 psi (Serlabo

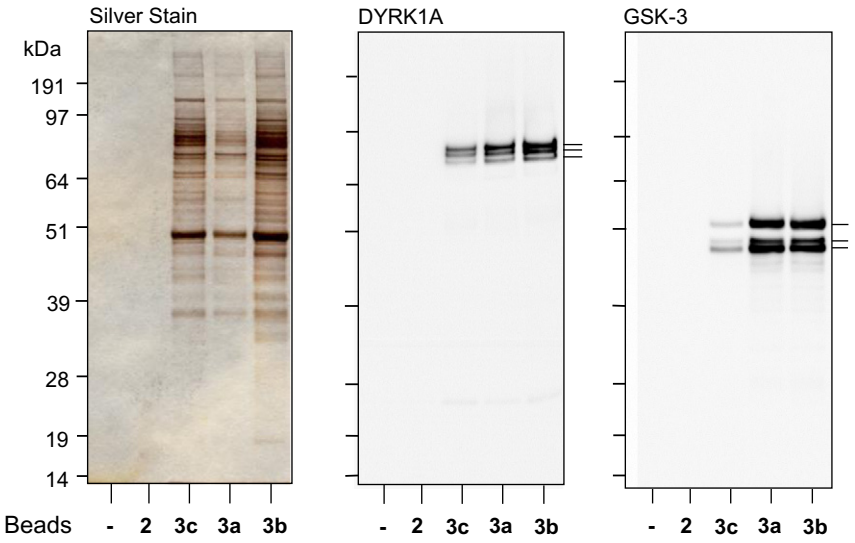


Fig. 2. Leucettine L41 binding proteins in mouse brain. Extracts, prepared from mouse brain, were loaded on control ethanolamine beads (−), linker **2**, inactive analogue **3c** beads, Leucettine L41 **3a** beads and Leucettine L41 **3b** beads. After extensive washing, the bound proteins were resolved by SDS-PAGE followed by silver staining (left panel) or Western blotting with anti-DYRK1A (central panel) and anti-GSK-3 antibodies (right panel).

Technologies France) using pre-packed column of silica gel 60 F 254 Merck equipped with a DAD UV/Vis 200–360 nm detector. Elemental analyses were performed on a Flash Microanalyzer EA1112 CHNS/O Thermo Electron in the "Centre Régional de Mesures Physiques de l'Ouest" (CRMPO, Rennes). Solvents were evaporated with a BUCHI rotary evaporator. All usual reagents and solvents were purchased from Acros, Aldrich Chimie, and Fluka France and were used without further purification. 2-[2-(2-Aminoethoxy) ethoxy]ethanol **3** was purchased from Aldrich Chimie France and compound **10a** was prepared according to our previous method described in literature [12].

*3.1.1.1. tert-Butyl N-(4-hydroxyphenyl)carbonate (**5**).* In a 250 mL two-necked round-bottomed flask provided with a magnetic stirrer and condenser, commercial 4-aminophenol **4** (4 g, 36.6 mmol) was solubilized in THF (100 mL) at room temperature. To this mixture, a solution of commercial di-*tert*-butyl dicarbonate (8 g, 36.6 mmol) in THF (50 mL) was added drop wise during 20 min. The reaction mixture was vigorously stirred at 25 °C during 24 h and was concentrated in a rotary evaporator under reduced pressure. The desired *tert*-butyl *N*-(4-hydroxyphenyl)carbamate **5** was obtained as white needles in quantitative yield (7.58 g) and was further used without purification. ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 1.44$ (s, 9H, CH_3), 6.63 (d, 2H, $J = 8.5$ Hz, H-2), 7.19 (d, 2H, $J = 7.9$ Hz, H-1), 8.96 (br s, 1H, OH), 9.04 (br s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6) $\delta = 28.09$, 78.49, 114.97, 120.06, 130.88, 152.41, 153.03. HRMS, $m/z = 232.0949$ found (calculated for $C_{11}H_{15}NO_3Na$, $[M + Na]^+$ requires 232.0949).

*3.1.1.2. 2-[2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy]ethyl-4-methylbenzenesulfonate (**6c**).* In a 100 mL two-necked round-bottomed flask provided with a magnetic stirrer and condenser, commercial tetraethyleneglycol **6b** (22 mL, 0.125 mol, 8 equiv.) was solubilized in 8 mL of dry THF. Then a solution of sodium hydroxide (1 g, 25.18 mmol, 1.6 equiv.) in deionized water (6 mL) was added in the reaction mixture followed by a drop wise addition of a solution of *para*-toluene sulfonylchloride (3 g, 15.73 mmol, 1 equiv.) in dry THF (20 mL). The reaction mixture was vigorously stirred at 0 °C during 2 h. After addition of cooled water (90 mL), the whole mixture was transferred into a separating funnel; after separating and extraction with methylene chloride (3 × 50 mL), the combined organic phases were dried over $MgSO_4$, filtered and the solvent of the filtrate was eliminated in a rotary evaporator under reduced pressure. The colorless oil was dried under high vacuum (10^{-3} Torr) at 25 °C for 1 h which gave the desired compound **6c** in 96% yield (5.26 g). This product **3c** was further used without purification. ^{1}H NMR (300 MHz, $CDCl_3$) $\delta = 2.45$ (s, 3H, CH_3), 3.78–3.53 (m, 14H),

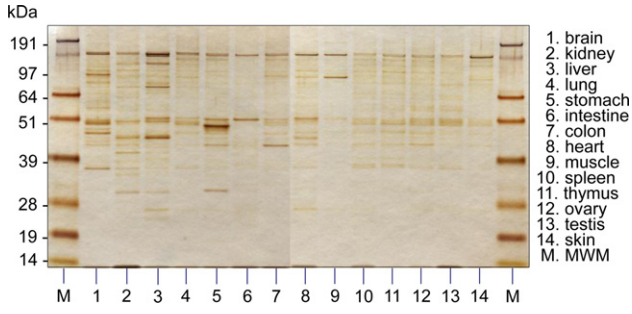


Fig. 3. Affinity chromatography purification of Leucettine L41 targets from various mouse tissues. Extracts of mouse tissues (brain, kidney, liver, lung, stomach, intestine, colon, heart, muscle, spleen, thymus, ovary, testis, skin) were prepared and loaded on immobilized Leucettine L41 **3a** (3 EG units linker). Beads were extensively washed and the bound proteins were resolved by SDS-PAGE followed by silver staining.

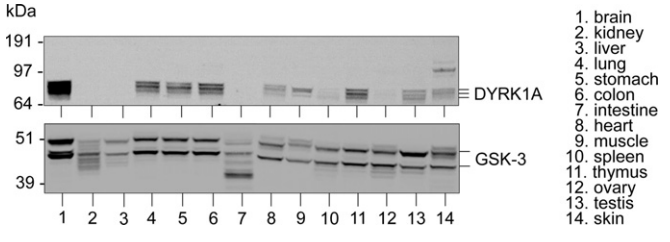


Fig. 4. Identification of affinity chromatography purified Leucettine L41 targets from various mouse tissues. Extracts of mouse tissues (brain, kidney, liver, lung, stomach, intestine, colon, heart, muscle, spleen, thymus, ovary, testis, skin) were prepared and loaded on immobilized Leucettine L41 **3a** (3 EG units linker). Beads were extensively washed and the bound proteins were resolved by SDS-PAGE followed by Western blotting using antibodies directed against DYRK1A and GSK-3.

4.16 (t, 2H, $J = 5.4$ Hz, H-10), 7.34 (d, 2H, $J = 8.6$, 0.6 Hz, H-1), 7.80 (d, 2H, $J = 8.3$ Hz, H-2). ^{13}C NMR (75 MHz, $CDCl_3$) $\delta = 21.59$, 61.60, 68.61, 69.27, 70.23, 70.37, 70.57, 70.64, 72.47, 127.91, 129.82, 132.88, 144.84. HRMS, $m/z = 371.1144$ found (calculated for $C_{15}H_{24}O_7SNa$, $[M + Na]^+$ requires 371.1140).

*3.1.1.3. tert-Butyl N-[4-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]phenyl]carbamate (**7a**).* In a 250 mL two-necked round-bottomed flask provided with a magnetic stirrer and condenser, *tert*-butyl *N*-(4-hydroxyphenyl)carbamate **5** (1 g, 4.78 mmol) was dissolved in dry acetonitrile (10 mL). To this solution was added successively at room temperature 2-[2-(2-chloroethoxy)ethoxy]ethanol **6a** (1.4 mL, 9.56 mmol, 2 equiv.) and potassium carbonate (1.98 g, 14.34 mmol, 3 equiv.). The reaction mixture was vigorously stirred at 81 °C during 48 h. After cooling down to room temperature, the solvent was eliminated in a rotary evaporator under reduced pressure and to the crude residue was added 50 mL of water. The whole mixture was transferred to a separating funnel; after separating and extraction with AcOEt (3×50 mL), the combined organic phases were dried over $MgSO_4$, filtered and the solvent of the filtrate was eliminated in a rotary evaporator under reduced pressure. The crude residue was submitted to purification by preparative chromatography (Combi Flash R_f 200 psi apparatus) on pre-packed column of silica gel 60F 254 Merck using a step-wise gradient of cyclohexane/AcOEt (100–50%). Pooling and evaporation of the eluent *in vacuum* gave the expected compound **7a** in 39% yield (636 mg) as mobile colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 1.48$ (s, 9H, CH_3), 3.77–3.53 (m, 10H, H-3, H-4, H-5, H-6, H-7), 3.81 (t, 2H, H-8), 6.34 (br s, 1H, NH), 6.82 (d, 2H, $J = 8.9$ Hz, H-2), 7.23 (d, 2H, $J = 8.5$ Hz, H-1). ^{13}C NMR (75 MHz, DMSO-d_6) $\delta = 28.35$, 61.66, 67.65, 69.73, 70.32, 70.75, 72.49 ($3 \times CH_3$), 114.98 (C-2), 120.44 (C-1), 131.78 (**C**Me_3), 153.20 (C–O), 154.65 (C–N), 171.20 (C=O). HRMS, $m/z = 364.1736$ found (calculated for $C_{17}H_{27}NO_6Na$, $[M + Na]^+$ requires 364.1736).

*3.1.1.4. tert-Butyl N-[4-[2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethoxy]phenyl]carbamate (**7b**).* In a 50 mL two-necked round-bottomed flask provided with a magnetic stirrer and condenser was added successively 2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethoxy]ethyl-4-methylbenzenesulfonate **6c** (3.03 g, 8.65 mmol, 1 equiv.), potassium carbonate (1.98 g, 12.97 mmol, 1.5 equiv.) and *tert*-butyl *N*-(4-hydroxyphenyl)carbamate **5** (1.99 g, 9.51 mmol, 1.1 equiv.) in 15 mL of dry THF. The reaction mixture was vigorously stirred at 80 °C during 48 h. After cooling down to room temperature, the solvent was eliminated in a rotary evaporator under reduced pressure and to the crude residue was added 50 mL of water. The whole mixture was transferred to a separating funnel; after separating and extraction with methylene chloride (2×100 mL), the combined organic phases were dried over $MgSO_4$, filtered and the solvent of the filtrate was eliminated in a rotary evaporator under reduced pressure. The crude residue was submitted to purification by preparative chromatography (Combi Flash R_f 200 psi apparatus) on pre-packed column of silica gel 60F 254 Merck using a step-wise gradient of cyclohexane/AcOEt (100–50%). Pooling and evaporation of the eluent *in vacuum* gave the expected compound **7b** in 68% yield (2.27 g) as mobile colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) $\delta = 1.51$ (s, 9H, $3 \times CH_3$), 3.62 (t, 2H, $J = 5.0$ Hz), 3.78–3.66 (m, 12H), 3.84 (t, 2H), 4.11 (t, 2H, $J = 8.6$, 6.0 Hz, H-10), 6.35 (br s, 1H, NH), 6.86 (d, 2H, $J = 9.0$ Hz, H-2), 7.25 (d, 2H, $J = 9.5$ Hz, H-1). HRMS, $m/z = 408.2000$ found (calculated for $C_{19}H_{31}NO_7Na$, $[M + Na]^+$ requires 408.1999).

*3.1.1.5. 2-[2-[2-[4-(tert-Butoxycarbonylamino)phenoxy]ethoxy]ethoxy]ethyl-4-methylbenzenesulfonate (**8a**).* In a 250 mL two-necked round-bottomed flask provided with a magnetic stirrer and condenser, a mixture of carbamate **7a** (2.3 g, 6.72 mmol) with 4-dimethylaminopyridine (136 mg, 1.34 mmol, 0.2 equiv.) and triethylamine (2.32 mL, 20.15 mmol, 3 equiv.) in methylene chloride (30 mL) was stirred at 0 °C during 30 min. To this cooled reaction mixture was added dropwise a solution of *p*-tolulenesulfonylchloride (2.65 g, 16.79 mmol, 2.5 equiv.) during 20 min. The reaction mixture was stirred vigorously at room temperature during 24 h. After elimination of the solvent in a rotary evaporator under reduced pressure, the crude residue was submitted to purification by preparative chromatography (Combi Flash R_f 200 psi apparatus) on pre-packed column of silica gel 60F 254 Merck using a step-wise gradient of cyclohexane/AcOEt (100–50%). Pooling and evaporation of the eluent *in vacuum* gave the expected compound **8a** in 75% yield (2.49 g) as mobile colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 1.43$ (s, 9H, $3 \times CH_3$), 2.35 (s, 3H, CH_3), 3.52–3.63 (m, 6H, H-4, H-5, H-6), 3.72 (t, 2H, $J = 5.5$ Hz, H-7), 3.99 (t, 2H, $J = 5.5$ Hz, H-3), 4.07 (t, 2H, $J = 7.5$ Hz, H-8), 6.35 (br s, 1H, NH), 6.76 (d, 2H, $J = 11$ Hz, H-2), 7.18 (d, 1H, $J = 10$ Hz, 1H, H-10), 7.26 (d, 2H, $J = 11$ Hz, H-1), 7.72 (d, 2H, $J = 11.0$ Hz, H-9). ^{13}C NMR (75 MHz, DMSO-d_6) $\delta = 21.62$ (CH_3), 28.35 ($3 \times CH_3$), 67.72 (CH_2), 68.70 (CH_2), 69.24 (CH_2), 69.82 (CH_2), 70.72 (CH_2), 70.77 (CH_2), 115.01 (CH, Ar), 120.43 (CH, Ar), 127.96 (CH, Ar), 129.82 (CH, Ar), 131.69, 132.96, 144.81 (C–S), 153.13 (C–O), 154.76 (C–N), 171.25 (C=O). HRMS, $m/z = 518.1826$ found (calculated for $C_{24}H_{33}NO_8Na$, $[M + Na]^+$ requires 518.1824).

*3.1.1.6. 2-[2-[2-[2-[4-(tert-Butoxycarbonylamino)phenoxy]ethoxy]ethoxy]ethoxy]ethyl-4-methylbenzenesulfonate (**8b**).* In a 50 mL two-necked round-bottomed flask provided with a magnetic stirrer and condenser, a solution of *p*-toluenesulfonylchloride (9.26 g, 48.61 mmol, 1.5 equiv.) in methylene chloride (10 mL) was added dropwise during 20 min. in a mixture at 0 °C of *tert*-butyl *N*-[4-[2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethoxy]phenyl]carbamate **7b** (12.52 g, 32.5 mmol, 1 equiv.) and triethylamine (9 mL, 97.52 mmol, 3 equiv.) in 30 mL of methylene chloride. The reaction mixture was stirred vigorously at room temperature during 24 h. After elimination of the solvent in a rotary evaporator under reduced pressure, the crude residue was submitted to purification by preparative chromatography (Combi Flash R_f 200 psi apparatus) on pre-packed column of silica gel 60F 254 Merck using a step-wise gradient of cyclohexane/AcOEt (100–50%). Pooling and evaporation of the eluent *in vacuum* gave the expected compound **8b** in 47% yield (8.24 g) as mobile colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 1.44$ (s, 9H), 2.38 (s, 3H, CH_3), 3.62–3.46 (m, 8H), 3.76–3.62 (m, 2H), 4.03–3.93 (m, 2H), 4.15–4.03 (m, 4H), 6.80 (d, 2H, $J = 8.5$ Hz, Ar), 7.33 (d, 2H, $J = 8.6$ Hz, Ar), 7.44 (d, 2H, $J = 8.4$ Hz, Ar), 7.75 (d, 2H, $J = 8.4$ Hz, Ar), 9.12 (br s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6) $\delta = 153.57$, 152.87, 144.82, 132.62, 132.31, 130.05, 127.55, 119.54, 114.36, 69.90, 69.81, 69.73, 69.63, 69.59, 68.94, 67.80, 67.15, 28.08, 26.27, 20.99 HRMS, $m/z = 562.2086$ found (calculated for $C_{26}H_{37}NO_9SNa$, $[M + Na]^+$ requires 562.2086).

*3.1.1.7. 4-[2-[2-(2-Azidoethoxy)ethoxy]ethoxy]aniline (**9a**).* In a 30 mL glass tube were placed successively the compound 2-[2-[2-[4-(*tert*-butoxycarbonylamino)phenoxy]ethoxy]ethoxy]ethyl-methylbenzenesulfonate **8a** (2.5 g, 5.04 mmol) and sodium azide (655 mg, 10.08 mmol, 2 equiv.) in 20 mL of a solution EtOH/H_2O (1:1). The glass tube was sealed with a snap cap and placed in the Anton Paar Monowave® 300 microwave cavity ($P = 800$ *W*). The stirred mixture was irradiated at 160 °C for 60 min. After microwave dielectric heating, the crude reaction mixture was allowed to cool down at room temperature and then was concentrated in a rotary evaporator under reduced pressure. After extraction with AcOEt (3×50 mL) and pooling, the combined phases were concentrated *in vacuum* and the resulting crude residue was dried

under high vacuum (10^{-3} Torr) at 25 °C for 1 h which gave the desired 4-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]aniline **9a** as a brown viscous oil in 90% yield (1.21 g). This product was further used without purification. ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 3.78$–3.63 (m, 6H, H-4, H-5, H-6), 3.39 (t, 2H, $J = 5.6$ Hz, H-7), 3.83 (t, 2H, $J = 5.6$ Hz, H-8), 4.06 (t, 2H, $J = 5.6$ Hz, H-3), 6.63 (d, 2H, $J = 8.9$ Hz, H-1), 6,77 (d, 2H, $J = 8.9$ Hz, H-2). ^{13}C NMR (75 MHz, DMSO-d_6) $\delta = 68.13$ (CH_2), 70.01 (CH_2), 70.06 (CH_2), 70.73 (CH_2), 70.72 (CH_2), 70.81 (CH_2), 115.87 (CH═, Ar), 116.33 (CH═, Ar), 140.19 (C–O), 151.91 (C–N). HRMS, $m/z = 289.1274$ found (calculated for $C_{12}H_{18}N_4O_3Na$, $[M + Na]^+$ requires 289.1276).

*3.1.1.8. 4-[2-[2-[2-(2-Azidoethoxy)ethoxy]ethoxy]ethoxy]aniline (**9b**).* In a 30 mL glass tube were placed successively the compound 2-[2-[2-[2-[4-(tert-butoxycarbonylamino)phenoxy]ethoxy]ethoxy] ethoxy]ethyl-4-methylbenzenesulfonate **8b** (2.5 g, 4.63 mmol, 1 equiv.) and and sodium azide (655 mg, 10.08 mmol, 2 equiv.) in 20 mL of a solution EtOH/H_2O (1:1). The glass tube was sealed with a snap cap and placed in the Anton Paar Monowave® 300 microwave cavity ($P = 800$ W). The stirred mixture was irradiated at 160 °C for 60 min. After microwave dielectric heating, the crude reaction mixture was allowed to cool down at room temperature and then, was concentrated in a rotary evaporator under reduced pressure. After extraction with AcOEt (3 × 50 mL) and pooling, the combined phases were concentrated *in vacuum* and the resulting crude residue was dried under high vacuum (10^{-3} Torr) at 25 °C for 1 h which gave the desired 4-[2-[2-[2-(2-azidoethoxy)ethoxy] ethoxy]ethoxy]aniline **9b** as a brown viscous oil in 95% yield (1.36 g). This product was further used without purification. ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 3.66$–3.29 (m, 12H), 3.71–3.67 (m, 2H), 3.95–3.84 (m, 2H), 6.49 (d, 2H, $J = 8.6$ Hz, Ar), 6.63 (d, 2H, $J = 8.6$ Hz, Ar). ^{13}C NMR (75 MHz, DMSO-d_{6v}) $\delta = 149.70$, 142.37, 115.25, 114.89, 69.82, 69.78, 69.75, 69.63, 69.19, 69.11, 67.49, 49.92. HRMS, $m/z = 333.1537$ found (calculated for $C_{14}H_{22}N_4O_4Na$, $[M + Na]^+$ requires 333.1538).

*3.1.1.9. (5Z) 5-Benzylidene-2-ethylsulfanyl-3,5-dihydro-imidazol-4-one (**10b**).* In a 30 mL glass tube were placed successively commercial thiohydantoin (1 g, 8.61 mmol), 0.96 mL of benzaldehyde **12b** (9.47 mmol, 1.1 equiv.), sodium acetate (777 mg, 9.47 mmol, 1.1 equiv.) and 4 mL of glacial acetic acid. The glass tube was sealed with a snap cap and placed in the Explorer® 24 CEM microwave cavity ($P = 300$ W). The stirred mixture was irradiated at 140 °C for 20 min under vigorous stirring. After microwave dielectric heating, the crude reaction mixture was allowed to cool down at room temperature after which 10 mL of deionized water were added in one portion. Then, the resulting mixture was triturated during 20 min. with slow stirring and yellow needles appeared in the suspension. The desired insoluble (5*Z*) 5-benzylidene-2-thioxo-imidazolidin-4-one **13b** was collected by filtration then washed with deionized water (2 mL) and dried under high vacuum (10^{-2} Torr) at 25 °C for 1 h. The product **13b** was obtained as yellow needles in 93% yield (1.63 g). Mp >250 °C. ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 6.48$ (s, 1H, CH═), 7.51–7.30 (m, 3H, H-3, H-4, H-5), 7.73 (d, 2H, $J = 4.1$ Hz, H-2, H-6), 12.15 (br s, 1H, NH), 12.38 (br s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6) $\delta = 111.50$ (**C**H═), 127.68 (C-1), 128.74 (C-3, C-5), 129.19 (C-4), 130.13 (C-2, C-6), 132.27 (C–N), 165.74 (C═O), 179.18 (C═S). HRMS, $m/z = 227.0251$ found (calculated for $C_{10}H_8N_2OSNa$, $[M + Na]^+$ requires 227.0250).

In a 50 mL two-necked round-bottomed flask provided with a magnetic stirrer and condenser were placed 0.70 mL of ethyl iodide (8.78 mmol, 1.1 equiv.) and potassium carbonate (551 mg, 3.99 mmol, 0.5 equiv.). To this mixture was added a solution of (5*Z*) 5-benzylidene-2-thioxo-imidazolidin-4-one **13b** (1.63 g, 7.98 mmol, 1 equiv.) in dry dimethylformamide (10 mL). The reaction mixture was heated at 60 °C under vigorous stirring during 12 h. After cooling down to room temperature, 30 mL of deionized water were added in one portion at 0 °C in the reaction mixture. The resulting suspension was stirred slowly at 0 °C over a period of 2 h and the desired insoluble (5*Z*) 5-benzylidene-2-ethylsulfanyl-1*H*-imidazol-4-one **10b** was collected by filtration and washed twice with deionized water (10 mL). Then, the compound **10b** was dried under high vacuum (10^{-2} Torr) at 25 °C for 1 h and was further used without purification. The product **10b** was obtained as yellow needles in 98% yield (1.81 g). Mp >250 °C. ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 1.39$ (t, 3H, CH_3), 3.28 (q, 2H, CH_2), 6.73 (s, 1H, CH═), 7.30–7.52 (m, 3H, H-3, H-4, H-5, Ar), 8.17 (d, 2H, H-2, H6, Ar), 11.78 (br s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6) $\delta = 14.51$ (CH_2), 24.20 (CH_3), 120.60 (**C**H═), 128.60 (C-3, C-5), 129.43 (C-4), 131.32 (C-2, C-6), 134.31 (C-1), 139.24 (C–N), 164.44 (C═O), 170.52 (C–S). HRMS, $m/z = 255.0569$ found (calculated for $C_{12}H_{12}N_2OSNa$, $[M + Na]^+$ requires 255.0568).

*3.1.1.10. (5Z) 2-[4-[2-[2-(2-Azidoethoxy)ethoxy]ethoxy]phenylamino]-5-(benzo[1,3]dioxol-5-ylmethylene)-3,5-dihydro-imidazol-4-one (**11a**).* In a 10 mL glass tube were placed successively the compound (5*Z*) 5-benzo[1,3]dioxol-5-ylmethylene-2-ethylsulfanyl-3,5-dihydro-imidazol-4-one **10a** (100 mg, 0.36 mmol, 1 equiv.) and 4-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]aniline **9a** (140 mg, 0.72 mmol, 1.4 equiv.). The glass tube was sealed with a snap cap and placed in the Explorer® 24 CEM microwave cavity ($P = 30$ W). The stirred mixture was irradiated at 160 °C for 30 min. After microwave dielectric heating, the crude reaction mixture was allowed to cool down at room temperature. The residue was dissolved in methylene chloride (5 mL) and was submitted to purification by preparative chromatography (Combi Flash R_f 200 psi apparatus) on pre-packed column of silica gel 60F 254 Merck using a step-wise gradient of CH_2Cl_2/MeOH (100–95%). Pooling and evaporation of the eluent *in vacuum* gave the desired azido compound **11a** in 18% yield (25.6 mg) as a yellowish powder. Mp >260 °C (decomposition). ^{1}H NMR (300 MHz, DMSO-d_6) $\delta = 3.39$ (t, 2H, $J = 5.4$ Hz), 3.68–3.54 (m, 6H), 3.74 (t, 2H, $J = 5.6$ Hz, H-13), 4.08 (t, 2H, $J = 4.8$ Hz, H-14), 6.05 (s, 2H, OCH_2O), 6.41 (s, 1H, CH═), 6.94 (dd, 3H, $J = 8.3$ Hz, H-7, H-6), 7.43 (d, 1H, $J = 8.2$ Hz, H-5), 7.64 (d, 2H, $J = 8.9$ Hz, H-8), 7.90 (s, 1H, H-2), 9.60 (br s, 1H, NH), 10.62 (br s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6) $\delta = 49.93$ (OCH_2O), 67.30 (CH_2), 68.98 (CH_2), 69.23 (CH_2), 69.65 (CH_2), 69.89 (CH_2), 108.37 (CH═), 109.17 (CH═), 113.96 (CH═), 114.64 (CH═), 120.98 (**C**H═C), 125.32 (**C**H═C), 129.91, 139.21, 146.99, 147.29, 154.29, 154.53, 170.34. HRMS, $m/z = 481.1837$ found (calculated for $C_{23}H_{24}N_6O_6Na$, $[M + Na]^+$ requires 481.1835).

*3.1.1.11. (5Z) 2-[4-[2-[2-[2-(2-Azidoethoxy)ethoxy]ethoxy]ethoxy] phenylamino]-5-(benzo[1,3]dioxol-5-ylmethylene)-3,5-dihydro-imidazol-5-one (**11b**).* In a 10 mL glass tube were placed successively the compound (5*Z*) 5-benzo[1,3]dioxol-5-ylmethylene-2-ethylsulfanyl-3,5-dihydro-imidazol-4-one **10a** (81 mg, 0.293 mmol, 1 equiv.) then 4-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethoxy]aniline **9b** (100 mg, 0.351 mmol, 1.2 equiv.) and 10 mL of hexane. The glass tube was sealed with a snap cap and placed in the Explorer® 24 CEM microwave cavity ($P = 300$ W). The stirred mixture was irradiated at 160 °C for 30 min. After microwave dielectric heating, the crude reaction mixture was allowed to cool down at room temperature. The residue was dissolved in methylene chloride (5 mL) and was submitted to purification by preparative chromatography (Combi Flash R_f 200 psi apparatus) on pre-packed column of silica gel 60F 254 Merck using a step-wise gradient of CH_2Cl_2/MeOH (100–95%). Pooling and evaporation of the eluent *in vacuum* gave the desired azido compound **11b** in 6% yield (8.8 mg) as a yellowish powder. Mp >260 °C (decomposition). ^{1}H NMR (300 MHz, DMSO-d_6)

δ = 3.63–3.39 (m, 12H), 3.81–3.67 (m, 3H), 4.15–3.98 (m, 2H), 6.04 (s, 2H), 6.41 (s, 1H, CH=), 7.06–6.86 (m, 3H), 7.43 (d, 1H, J = 8.2 Hz, Ar), 7.64 (d, 2H, J = 8.9 Hz, Ar), 7.90 (s, 1H, H-2), 9.60 (br s, 1H, NH), 10.62 (br s, 1H, NH). HRMS, m/z = 521.2025 found (calculated for $C_{25}H_{30}N_4O_7Na$, $[M+Na]^+$ requires 521.2012).

*3.1.1.12. (5Z) 2-[4-[2-[2-(2-Azidoethoxy)ethoxy]ethoxy]anilino]-4-benzylidene-3,5-dihydro-imidazol-5-one (**11c**).* In a 10 mL glass tube were placed successively the compound (5*Z*) 5-benzylidene-2-ethylsulfanyl-3,5-dihydro-imidazol-4-one **10b** (100 mg, 0.43 mmol, 1 equiv.) and 4-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]aniline **9a** (137 mg, 0.52 mmol, 1.2 equiv.). The glass tube was sealed with a snap cap and placed in the Explorer® 24 CEM microwave cavity (P = 300 W). The stirred mixture was irradiated at 160 °C for 30 min. After microwave dielectric heating, the crude reaction mixture was allowed to cool down at room temperature. The residue was dissolved in methylene chloride (5 mL) and was submitted to purification by preparative chromatography (Combi Flash R_f 200 psi apparatus) on pre-packed column of silica gel 60F 254 Merck using a step-wise gradient of CH_2Cl_2/MeOH (100–95%). Pooling and evaporation of the eluent *in vacuum* gave the desired azido compound **11c** in 6% yield (11.3 mg) as a yellowish powder. Mp >260 °C (decomposition). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 3.38 (t, 2H, J = 4.8 Hz), 3.70–3.52 (m, 6H), 3.76 (t, 2H, J = 4.3 Hz), 4.09 (t, 2H, J = 4.8 Hz), 6.44 (s, 1H, CH=), 6.98 (d, 2H, J = 8.8 Hz, H-8), 7.28 (d, 1H, J = 7.2 Hz, 1H), 7.39 (dd, 2H, J = 7.4 Hz, H-3, H-5), 7.69 (d, 2H, J = 8.3 Hz, H-7), 8.07 (d, 2H, J = 7.5 Hz, H-6, H-2), 9.69 (br s, 1H, NH), 10.67 (br s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6) δ = 49.93, 67.27, 68.97, 69.23, 69.66, 69.89, 113.49, 114.70, 121.03, 127.62, 128.38, 130.08, 131.84, 135.54, 140.81, 154.33, 155.06, 170.44. HRMS, m/z = 459.1759 found (calculated for $C_{22}H_{24}N_6O_4Na$, $[M+Na]^+$ requires 459.1759).

*3.1.1.13. (5Z) 2-[4-[2-[2-(2-Aminoethoxy)ethoxy]ethoxy]phenylamino]-5-(benzo[1,3]dioxol-5-ylmethylene)-3,5-dihydro-imidazol-4-one (**3a**).* In a 10 mL round-bottomed flask provided with a magnetic stirrer and condenser, a mixture of 175 mg (2 equiv.) of triphenylphosphine (1.5 mmol/g) bounded on polymer (200–400 Mesh, Sigma) and (5*Z*) 2-[4-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]phenylamino]-5-benzo[1,3]dioxol-5-ylmethylene-3,5-dihydro-imidazol-4-one **11a** (125 mg, 0.26 mmol) in 5 mL of THF was heated under reflux during 24 h in a thermostated oil bath. To this mixture was added deionized water (0.5 mL) and heating was pursued at 81 °C for 24 h under magnetic stirring. After cooling down to room temperature, insoluble triphenylphosphine oxide bound on polymer was collected by filtration then washed with ethanol (2 × 10 mL). The resulting filtrate was concentrated in a rotary evaporator under reduced pressure and the crude residue was dried under high vacuum (10^{-3} Torr) at 25 °C for 1 h which gave a yellowish solid compound **3a** in 75% yield (88.6 mg). This product **3a** was further used without purification for immobilization on Sepharose beads. Mp >260 °C (decomposition). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 3.40 (t, 2H, J = 5.1 Hz), 3.67–3.54 (m, 6H), 3.74 (t, 2H, J = 4.6 Hz, H-13), 4.07 (t, 2H, J = 4.6 Hz, H-14), 6.05 (s, 2H, OCH_2O), 6.40 (s, 1H, CH=), 6.94 (dd, 3H, J = 8.5 Hz, H-6, H-8), 7.44 (d, 1H, J = 7.9 Hz, H-5), 7.63 (d, 2H, J = 9 Hz, H-7), 7.87 (s, 1H, H-2), 9.61 (br s, 1H, NH), 9.72 (br s, 2H, NH_2), 10.62 (br s, 1H, NH). HRMS, m/z = 455.1931 found (calculated for $C_{23}H_{27}N_4O_6$, $[M+H]^+$ requires 455.1930).

*3.1.1.14. (5Z)-2-[4-[2-[2-[2-(2-Aminoethoxy)ethoxy]ethoxy]ethoxy]phenylamino]-5-(benzo[1,3]dioxol-5-ylmethylene)-3,5-dihydro-imidazol-4-one (**3b**).* In a 10 mL round-bottomed flask provided with a magnetic stirrer and condenser, a mixture of 187 mg (1.5 equiv.) of triphenylphosphine (1.5 mmol/g) bounded on polymer (200–400 Mesh, Sigma) and (5*Z*) 2-[4-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]phenylamino]-5-benzo[1,3]dioxol-5-ylmethylene-3,5-dihydro-imidazol-5-one **11b** (98 mg, 0.187 mmol) in 5 mL of THF was heated under reflux during 24 h in a thermostated oil bath. To this mixture was added deionized water (0.5 mL) and heating was pursued at 81 °C for 24 h under magnetic stirring. After cooling down to room temperature, insoluble triphenylphosphine oxide bound on polymer was collected by filtration then washed with ethanol (2 × 10 mL). The resulting filtrate was concentrated in a rotary evaporator under reduced pressure and the crude residue was dried under high vacuum (10^{-3} Torr) at 25 °C for 1 h which gave a yellowish solid compound **3b** in 75% yield (73 mg). This product **3b** was further used without purification for immobilization on Sepharose beads. Mp >260 °C (decomposition). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 4.00–3.05 (m, 12H), 4.26–4.03 (m, 4H), 6.11 (s, 2H, OCH_2O), 6.46 (s, 1H, CH=), 6.82–7.08 (m, 3H, Ar), 7.47 (d, 1H, J = 8.3 Hz, Ar), 7.72 (d, 2H, J = 8.4 Hz, Ar), 7.94 (s, 1H), 9.62 (br s, 1H, NH), 9.74 (br s, 2H, NH_2), 10.66 (br s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6) δ = 154.16, 147.30, 146.87, 132.12, 129.94, 125.18, 120.85, 114.62, 109.06, 108.38, 101.06, 69.85, 69.74, 69.68, 69.53, 68.94, 67.28. HRMS, m/z = 521.2025 found (calculated for $C_{25}H_{30}N_4O_7Na$, $[M+H]^+$ requires 521.2012).

*3.1.1.15. (5Z) 2-[4-[2-[2-(2-Aminoethoxy)ethoxy]ethoxy]anilino]-4-benzylidene-3,5-dihydro-imidazol-5-one (**3c**).* In a 10 mL round-bottomed flask provided with a magnetic stirrer and condenser, a mixture of 100 mg (2 equiv.) of triphenylphosphine (1.5 mmol/g) bounded on polymer (200–400 Mesh, Sigma) and (5*Z*) 2-[4-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]anilino]-4-benzylidene-3,5-dihydro-imidazol-5-one **11c** (42 mg, 0.096 mmol) in 5 mL of THF was heated under reflux during 24 h in a thermostated oil bath. To this mixture was added deionized water (0.5 mL) and heating was pursued at 81 °C during 24 h under magnetic stirring. After cooling down to room temperature, insoluble triphenylphosphine oxide bound on polymer was collected by filtration then washed with ethanol (2 × 10 mL). The resulting filtrate was concentrated in a rotary evaporator under reduced pressure and the crude residue was dried under high vacuum (10^{-3} Torr) at 25 °C for 1 h which gave a yellowish solid compound **3c** in 76% yield (32 mg). This product **3c** was further used without purification for immobilization on Sepharose beads. Mp >260 °C (decomposition). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 3.75–3.56 (m, 8H), 3.81 (t, 2H, J = 4.6 Hz), 4.16 (t, 2H, J = 5.0 Hz), 6.48 (s, 1H, CH=), 7.04 (d, 2H, J = 9.1 Hz, H-8), 7.33 (d, 1H, J = 7.3 Hz, H-4), 7.47 (dd, 2H, J = 7.6 Hz, H-3, H-5), 7.78 (d, 2H, J = 8.9 Hz, H-7), 8.11 (d, 2H, J = 7.6 Hz Hz, H-2, H-6), 9.60 (br s, 1H, NH), 9.71 (br s, 2H, NH_2), 10.51 (br s, 1H, NH). HRMS, m/z = 433.1850 found (calculated for $C_{22}H_{26}N_4O_4Na$, $[M+H]^+$ requires 433.1860).

3.2. Biology

3.2.1. Buffers

Bead buffer: 50 mM Tris (pH 7.4), 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40 and protease inhibitor cocktail.

Blocking buffer: 1 M ethanolamine in coupling buffer, pH 8.0.

Coupling buffer: 0.1 M $NaHCO_3$ and 0.2 M NaCl, pH 8.3.

Homogenization buffer: 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 25 mM Mops (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylphosphate disodium and protease inhibitor cocktail.

Washing buffer: 0.1 M CH_3COONa, pH 4.

All chemicals were purchased from Sigma, unless otherwise stated and the protease inhibitor cocktail was from Roche.

3.2.2. Antibodies

Anti-DYRK1A (H00001859-M01, 1:1000) and anti-GSK-3 α/β (KAM-ST002E, 1:1000) were obtained from Interchim and Stessgen, respectively.

3.2.3. Affinity chromatography on immobilized Leucettines

3.2.3.1. Preparation of Leucettine agarose beads. CNBr-activated sepharose 4B (Sigma) was swollen in cold 1 mM HCl for 30 min. Beads were then activated with coupling buffer. Leucettine L41 or analogue with a linker were synthesized as described above and were coupled overnight under constant rotation at room temperature. After removal of the supernatant, residual active sites were quenched using blocking buffer for 2 h 30 min under constant rotation at room temperature. Beads were washed with coupling buffer, washing buffer and bead buffer and brought to a 20% suspension in bead buffer. They were stored at 4 °C until further use. Ethanolamine beads were used as controls.

3.2.3.2. Preparation of brain extracts. Brains were obtained from mice at the animal facility of the University of Rennes and snap-frozen until further use. Tissues were weighed, homogenized and sonicated in homogenization buffer (5 mL/g of material). Homogenates were centrifuged for 15 min at 17,000×*g* at 4 °C. The supernatant was recovered, assayed for protein content (BioRad Protein Assay) and kept at −80 °C until use.

3.2.3.3. Preparation of extracts of other tissues. The different mice tissues (brain, kidney, liver, lung, heart, stomach, intestines, colon, muscle, spleen, ovary, thymus and skin) were obtained from the University of Rennes animal facility and snap-frozen until further use. Tissues were weighed, homogenized and sonicated in homogenization buffer (5 mL/g of material for all tissues except 10 mL/g for kidney and liver). Homogenates were centrifuged for 15 min at 17,000×*g* at 4 °C. The supernatant was recovered, assayed for protein content (BioRad Protein Assay) and kept at −80 °C until use.

3.2.3.4. Affinity chromatography of Leucettine L41-interacting proteins. Just before use, 10 μL of packed beads were washed with 1 mL of bead buffer and resuspended in this buffer. After a brief spin at 17,000×*g*, the tissue extract supernatant (1 or 2 mg of total protein) was loaded on 10 μL of packed beads and the volume was adjusted to 1 mL with bead buffer. The tubes were rotated at 4 °C for 30 min. After a brief spin at 10,000×*g* and removal of the supernatant, the beads were washed 4 times with bead buffer before addition of 80 μL of 2× LDS sample buffer (Invitrogen) and 200 mM DTT. Following heat denaturation for 3 min, the bound proteins were analyzed by SDS-PAGE and Western blotting or silver staining as described below.

3.2.4. Electrophoresis and Western blotting

The proteins bound to the matrices were separated by 10% NuPAGE pre-cast Bis–Tris polyacrylamide mini gel electrophoresis (Invitrogen) with MOPS-SDS running buffer followed by silver staining (GE Healthcare Life Sciences, PlusOne Silver Staining Kit Protein, 17-1150-01) or immunoblotting analysis. Proteins were transferred to 0.45 μm nitrocellulose filters (Whatman). These were blocked with 5% low fat milk in Tris-buffered saline/Tween 20 and incubated overnight at 4 °C with antibodies. Appropriate secondary antibodies conjugated to horseradish peroxidase (BioRad) were added to visualize the proteins using the Enhanced Chemiluminescence reaction (ECL, Amersham).

3.2.5. Protein kinase assays

3.2.5.1. Buffer D. $MgCl_2$ (10 mM), 1 mM EGTA (ethyleneglycoltetraacetic acid), 1 mM DTT (dithiothreitol), 25 mM Tris/HCl, (50 μg/mL heparin).

Kinase activities were assayed in Buffer D (+0.5 mg BSA/mL + 1 mM DTT, except for CDK2), at 30 °C, at a final ATP concentration of 15 μM. Blank values were subtracted and activities expressed in % of the maximal activity, i.e. in the absence of inhibitors. Controls were performed with appropriate dilutions of DMSO. The GS-1, CKS, CDK7/9 tide and RS peptide substrates were obtained from Proteogenix (Oberhausbergen, France).

3.2.5.2. CDK1/cyclin B (M phase starfish oocytes, native), CDK2/cyclin E and CDK5/p25 (human, recombinant). CDK1/cyclin B (M phase starfish oocytes, native), CDK2/cyclin E and CDK5/p25 (human, recombinant) were prepared as previously described [15]. Their kinase activity was assayed in buffer D (+0.5 mg BSA /mL + 1 mM DTT, except for CDK2), with 1 mg histone H1/mL, in the presence of 15 μM [γ-^{33}P] ATP (3000 Ci/mmol; 10 mCi/mL) in a final volume of 30 μL. After 30 min incubation at 30 °C, the reaction was stopped by harvesting onto P81 phosphocellulose papers (Whatman) using a FilterMate harvester (Packard) and was washed in 1% phosphoric acid. Scintillation fluid was added and the radioactivity measured in a Packard counter.

3.2.5.3. CDK9/cyclin T (human, recombinant, expressed in insect cells). CDK9/cyclin T (human, recombinant, expressed in insect cells) was assayed as described for CDK1/cyclin B, but using CDK7/9 tide (YSPTSPSYSPTSPSYSPTSPSKKKK) (8.1 μg/assay) as a substrate.

3.2.5.4. GSK-3α/β (porcine brain, native). GSK-3α/β (porcine brain, native) was assayed, as described for CDK1 but using a GSK-3 specific substrate (GS-1: YRRAAVPPSPSLSRHSSPHQSpEDEEE) (pS stands for phosphorylated serine) [34].

3.2.5.5. CK1δ/ε (porcine brain, native). CK1δ/ε (porcine brain, native) was assayed as described for CDK1 but using 25 μM CKS peptide (RRKHAAIGpSAYSITA), a CK1-specific substrate [35].

3.2.5.6. DYRK1A, 1B, 2 and 3 (Human, recombinant, expressed in E. coli as GST fusion proteins) and CLK1, 2, 3 and 4 (mouse, recombinant, expressed in E. coli as GST fusion proteins). DYRK1A, 1B, 2 and 3 (Human, recombinant, expressed in *E. coli* as GST fusion proteins) and CLK1, 2, 3 and 4 (mouse, recombinant, expressed in E. coli as GST fusion proteins) were purified by affinity chromatography on glutathione-agarose and assayed as described for CDK1/cyclin B with RS peptide (GRSRSRSRSRSR) (1 μg/assay) as a substrate.

4. Discussion

In this article we report on the synthesis and affinity chromatography use of immobilized Leucettines. Leucettines constitute a promising class of kinase inhibitors in various therapeutic areas, with particular focus on Alzheimer's disease and Down syndrome at the moment [12,13]. It is therefore particularly important to develop methods allowing the identification of the primary and secondary targets of these molecules. Immobilization of Leucettines through a polyethylene linker was chosen as one method to analyze their selectivity since this approach has yielded a wealth of information when applied to other kinase inhibitors [14–17]. In addition as we had co-crystal structures of Leucettine L41 with several kinases available [12,13], it was possible to rationally decide where the linker should be attached to Leucettines without preventing their interaction with kinase targets. This is in fact demonstrated by the fact that Leucettine L41 without (**1a**) or with 4 EG (**3b**) display essentially the same kinase inhibitory selectivity profiles and potency (Table 2). However this method excludes potential targets that might interact with Leucettines just at the site

where the linker is attached. It would therefore be of interest to immobilize Leucettines at some other sites as well, such as in the methylenedioxy moiety. This might uncover yet undetected targets. This possibility is supported by the fact that the kinase inactive Leucettine **1b** shows modest but detectable activity when a PEG arm is attached (**3c**) (Table 2). In addition immobilized **3c** binds proteins including DYRK1A and GSK-3 (Fig. 2). These results suggest some interaction directly with the linker itself or through physico-chemical properties changes brought about in this area by the linker attachment. Nevertheless the specificity in binding to Leucettine beads was demonstrated by the fact that excess free ligand added to the cellular or tissue extracts prior to and during binding to immobilized Leucettine beads, resulted in reduced binding of the targets [13]. Another drawback of the affinity chromatography approach is the fact that it favors detection of the most abundant targets. Nevertheless all the identified targets found by affinity chromatography on immobilized Leucettines were also identified by a competitive affinity method (based on binding competition with immobilized broad spectrum kinase inhibitors which are able to concentrate most of the expressed kinome from any cell or tissue extract) [13]. Altogether, we believe we have established a solid synthesis protocol to prepare Leucettines for immobilization onto agarose beads, thereby providing a very useful reagent to purify, concentrate and identify Leucettines' targets from any biological source. As an illustration we have shown here that the expression pattern of Leucettine L41 in mouse tissues varies considerably from one organ to the other. Our aim is to develop this method further and to apply it to the most disease-relevant Leucettines with appropriate target tissue and cell samples.

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