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# Design, Synthesis and Preliminary Biological Evaluation of Rivastigmine-INDY Hybrids as Multitarget Ligands Against Alzheimer's Disease by Targeting Butyrylcholinesterase and DYRK1A/CLK1 Kinases

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Mihaela-Liliana Tintas, Ludovic Peauger, Anaïs Barré, Cyril Papamicael, Thierry Besson, et al.. Design, Synthesis and Preliminary Biological Evaluation of Rivastigmine-INDY Hybrids as Multitarget Ligands Against Alzheimer's Disease by Targeting Butyrylcholinesterase and DYRK1A/CLK1 Kinases. RSC Medicinal Chemistry, In press, 10.1039/D3MD00708A . hal-04478385

**HAL Id: hal-04478385**

**<https://hal.science/hal-04478385v1>**

Submitted on 26 Feb 2024

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# RSC Medicinal Chemistry

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This article can be cited before page numbers have been issued, to do this please use: M. TINTAS, L. Peauger, A. Barré, C. Papamicael, T. Besson, J. Sopkova de Oliveira Santos, V. Gembus and V. Levacher, *RSC Med. Chem.*, 2024, DOI: 10.1039/D3MD00708A.



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## ARTICLE

## Design, Synthesis and Preliminary Biological Evaluation of Rivastigmine-INDY Hybrids as Multitarget Ligands Against Alzheimer's Disease by Targeting Butyrylcholinesterase and DYRK1A/CLK1 Kinases†

Received 00th January 20xx,  
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Based on a multitarget approach implementing rivastigmine-INDY hybrids **1**, we identified a set of pseudo-irreversible carbamate-type inhibitors of *eq*BuChE that, after carbamate transfer at the active site serine residue, released the corresponding INDY analogues **2** endowed with *h*DYRK1A/*h*CLK1 kinases inhibitory properties. A SAR study and molecular docking investigation of both series of compounds **1** and **2** revealed that appropriate structural modifications at the carbamate moiety and at the *N*-appendage of the benzothiazole core led to potent and selective *eq*BuChE inhibitors with IC<sub>50</sub> up to 27 nM and potent *h*DYRK1A and *h*CLK1 inhibitors with IC<sub>50</sub> up to 106 nM and 17nM respectively. Pleasingly, identification of the matched pair of compounds **1b/2b** with a good balance between inhibition of *eq*BuChE and *h*DYRK1A/*h*CLK1 kinases (IC<sub>50</sub> = 68 nM and IC<sub>50</sub> = 529/54 nM, respectively) further validated our multitarget approach based on a sequential mechanism of action. In addition, target compound **1b** exhibited a suitable ADMET profile, including good brain permeability and high stability in PBS, encouraging further biological investigation as a drug candidate.

### Introduction

Alzheimer's Disease (AD), the main cause of dementia in the elderly and affecting more than 55 million people worldwide, is a multifactorial disorder marked by a progressive decline in learning ability and the impairment of other cognitive functions<sup>1</sup>. Pathologically, AD is most often associated with the abnormal formation of extracellular amyloid  $\beta$ -plaques and intracellular neurofibrillary tangles, neuronal oxidative stress and cholinergic dysfunction<sup>2,3</sup>. Although decades of intensive research and development have been devoted to designing curative treatments, all drug candidates aiming at slowing down the progression or at delaying the onset of AD failed to demonstrate their effectiveness in advanced clinical trials.<sup>4</sup> Although the FDA recently approved Lecanemab for AD, a monoclonal antibody that reduces brain amyloid levels in early AD patients and lead in a moderate reduction in cognitive and functional decline,<sup>5</sup> most of the treatments currently available aim to alleviate symptoms associated with cognitive deficits by restoring physiological levels of acetylcholine.<sup>6,7</sup> There is a broad scientific consensus that the current lack of effective

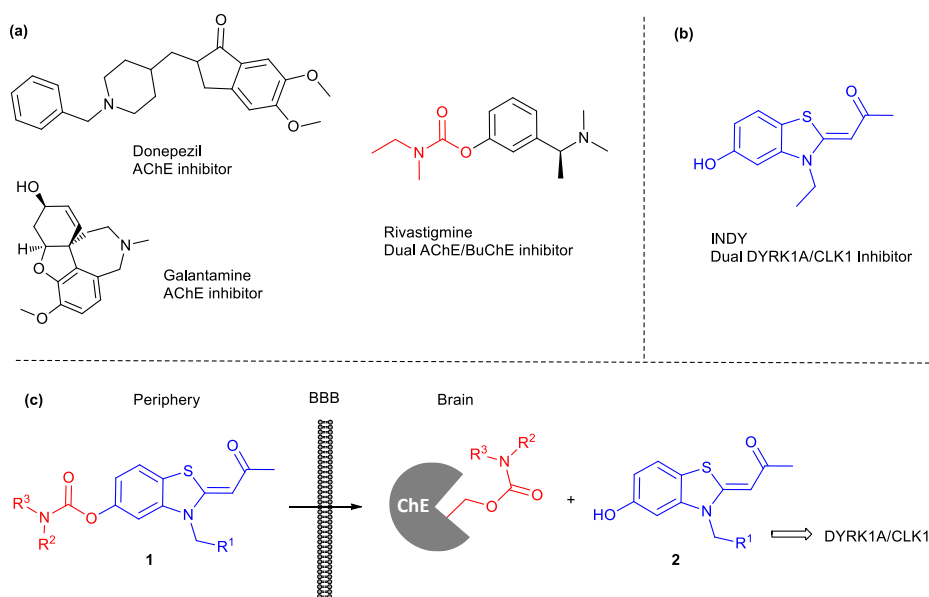
treatments might be due to the multifactorial nature of AD which makes mainstream single-target drugs (the "one compound–one target" approach) ineffective. In the last two decades, multitarget agents designed to inhibit more than one specific protein involved in the development of AD, have emerged as a new promising approach, as they take into consideration the complex pathophysiology of AD.<sup>8</sup> Among multi-target drug candidates, those designed to alter the pathogenesis of AD by preventing the formation of  $\beta$ -amyloid plaques and/or neurofibrillary tangles while restoring the cholinergic balance in AD patients are highly sought after.<sup>9</sup> The three cholinesterase inhibitors currently used in the treatment of AD differ in selectivity for acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Whereas donepezil and galantamine are AChE selective inhibitors, rivastigmine displays a dual inhibition of both AChE and BuChE (Fig. 1a). It is also worth noting that rivastigmine is a carbamate-type cholinesterase inhibitor that covalently binds to enzymes *via* carbamoylation of the serine residue at the active site of cholinesterases. Early research in AD treatments focused on selective AChE inhibitors based on the rationale that AChE largely prevails in healthy subjects, ruling out BuChE as a key player in regulating ACh levels. However, there is a common understanding that both AChE and BuChE might be involved in regulating ACh levels in the brain of AD patients since in advanced stages of the disease, AChE activity drops by at least half while BuChE activity progressively increases up to 120%.<sup>10-14</sup> Besides cholinergic benefits, BuChE is thought to be implicated in the aggregation of amyloid- $\beta$  peptide.<sup>15</sup>

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†Electronic Supplementary Information (ESI) available: <sup>1</sup>H and <sup>13</sup>C NMR spectra, HRMS spectra **1b-p**, amino-acid sequence alignment of *eq*BuChE and *h*BuChE. See DOI: 10.1039/x0xx00000x



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DOI: 10.1039/D3MD00708A

Fig.1 (a) Currently marketed cholinesterase inhibitors: donepezil, galantamine and rivastigmine; (b) INDY: a dual DYRK1A/CLK1 inhibitor; (c) Design of rivastigmine-INDY hybrids **1**.

Additionally, BuChE inhibitors are believed to induce fewer side effects than AChE inhibitors.<sup>16</sup> As a result, there has been a strong and growing interest these in recent years in developing new dual AChE/BuChE inhibitors as well as highly selective BuChE inhibitors.<sup>17-19</sup>

In addition to cholinergic dysfunction, the overexpression of protein kinases in AD patient brains entails abnormal kinase activity that has an impact on the AD pathophysiology making protein kinases interesting targets for drug discovery in AD.<sup>20</sup> In particular, dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) and Cdc2-like kinase 1 (CLK1) are both involved in the regulation of alternative pre-mRNA splicing; a process that, when deregulated, plays a key role in AD pathophysiology.<sup>21</sup> Additionally, DYRK1A is also involved in the formation of neurofibrillary tangles through hyperphosphorylation of Tau protein.<sup>22</sup> The major role played by these two kinases in the pathogenesis of AD prompted the scientific community to develop new dual DYRK1A/CLK1 inhibitors. Of the many synthetic dual DYRK1A/CLK1 inhibitors reported in the literature<sup>23,24</sup> our attention was drawn to INDY,<sup>25</sup> which is a simple benzothiazole scaffold showing a potent inhibitory activity and good selectivity against both DYRK1A/CLK1 kinases (Fig. 1b).

A survey of the literature revealed that cholinesterases are by far the most widely investigated biological targets in the search for multitarget ligands for AD, while highlighting that the development of dual cholinesterase-kinase inhibitors represents a largely unexplored avenue.<sup>8</sup> As part of a research and development program focusing on the discovery of new polypharmacological drug candidates for AD, we report herein on the design, synthesis and preliminary biological evaluation of a novel series of anti-AD hybrids **1** by merging the aryl carbamate moiety of rivastigmine and the benzothiazole

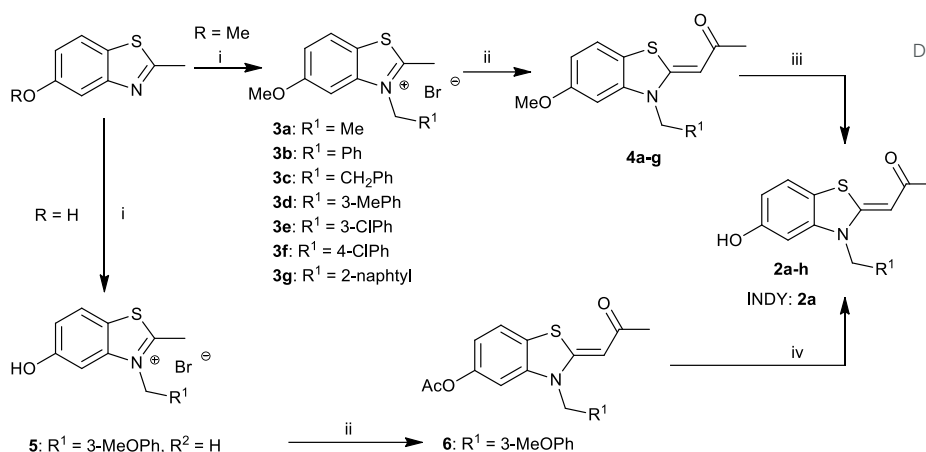
scaffold of INDY. According to the scenario depicted in Fig. 1C, after crossing the blood–brain barrier (BBB), the newly designed carbamoylated INDY derivatives **1** are presumed to initially inhibit central cholinesterases by carbamylation of the catalytic triad serine with simultaneous release of INDY analogues **2** that can in turn interact with DYRK1A/CLK1 kinases. In this paper, we report on the design and synthesis of these new carbamoylated INDY derivatives **1** and a Structure–Activity Relationship (SAR) study highlighted from *in vitro* biological evaluation towards BuChE/AChE and DYRK1A/CLK1 kinases.

## Results and Discussion

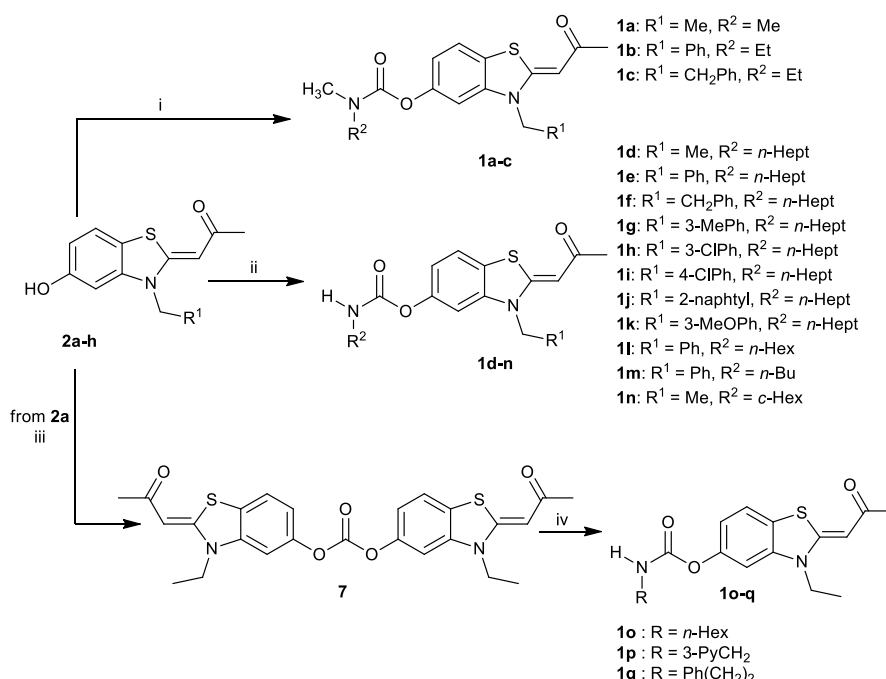
### Synthesis of carbamoylated INDY derivatives 1a-q

A set of carbamoylated INDY derivatives **1a-q** were prepared to undertake a SAR study that focuses on the influence of the *N*-substituent ( $R^1$ ) and that of the carbamate on their inhibitory properties. The overriding goal of this SAR study was to identify a multitarget drug candidate that strikes a fair balance between good inhibitory properties towards cholinesterases (BuChE and/or AChE) and kinases (DYRK1A and/or CLK1) while also ensuring good BBB permeability.

The required *N*-alkylated 5-hydroxy INDY analogues **2b-h** were prepared according to a modified procedure that was previously reported for the synthesis of INDY **2a**<sup>26</sup> (Scheme 1). Thus, commercially available 5-methoxy-2-methylbenzothiazole was first reacted with alkyl halides to afford the corresponding *N*-substituted benzothiazolinium salts **3a-g** in good to excellent yields (64-96%) which were afterwards subjected to acetic anhydride to give the corresponding enaminones **4a-g** in moderate to good yields (32-88%). Subsequent *O*-demethylation was achieved under classical conditions using boron tribromide to furnish INDY **2a** and its analogues **2b-g** with yields ranging from 31-95%. An alternative



**Scheme 1** i) CH<sub>3</sub>CH<sub>2</sub>I, 80°C, 5 days, sealed tube (96%) for **3a** or R<sup>1</sup>CH<sub>2</sub>Br, 85°C, 18h (64-92%) for **3b-g**, 88% for **5**; ii) Ac<sub>2</sub>O, NEt<sub>3</sub>, CH<sub>3</sub>CN, sealed tube, 85°C, 18h (32-88%) for **4a-g** (72%) for **6**; iii) BBr<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 0°C to 20°C, 24h (95%) for **2a** or 2h (31-92%) for **2b-g**; iv) NaOH, MeOH, 65°C, 15min for **2h** (68%).



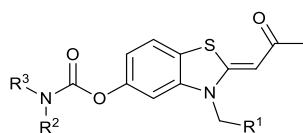
**Scheme 2** i) (CH<sub>3</sub>)<sub>2</sub>NCOCl for **1a** from **2a** or (CH<sub>3</sub>)(C<sub>2</sub>H<sub>5</sub>)NCOCl for **1b,c** from **2b,c**, K<sub>2</sub>CO<sub>3</sub>, DMF, 20°C, 18h (31-78%); ii) *n*-Heptyl or *n*-hexyl or *n*-butyl or cyclohexyl isocyanate, DMF or CH<sub>2</sub>Cl<sub>2</sub>, NEt<sub>3</sub>, sealed tube, 20-65°C, 18h, for **1d-k** (12-95%), **1l** (63%) and **1m** (82%), **1n** (56%); iii) NaH, DSC, DMF, 20°C, 1h (81%); iv) Hexylamine or 2-picolylamine or 2-phenethylamine, CH<sub>2</sub>Cl<sub>2</sub>, NEt<sub>3</sub>, 20°C, respectively 4h30 for **1o** (78%), 2h for **1p** (38%), 3h for **1q** (19%).

synthetic route was investigated for the preparation of the INDY analogue **2h** to circumvent the *O*-demethylation step that turned out to be trickier to perform owing to the presence an additional methoxy group on the *N*-benzyl appendage (R<sup>1</sup>). It brings into play an initial quaternization by 3-methoxybenzyl bromide of commercially available 2-methylbenzothiazol-5-ol to yield *N*-alkylated benzothiazol-5-ol **5** (88%). The so-obtained benzothiazolinium salt **5** was further reacted with acetic anhydride to give compound **6** (72%). The desired INDY analogue **2h** was successfully obtained after an ultimate step of deacetylation under basic conditions in 68% yield.

In a last stage, various carbamate groups were installed on INDY analogues **2a-h** (Scheme 2) to probe the influence of the

carbamate moiety of the so-obtained rivastigmine-INDY hybrids **1a-q** on their inhibitory potency towards both BuChE and AChE. In a first approach, *N,N*-dialkylcarbamate derivatives **1a-c** were prepared by reacting **2a-c** with the corresponding commercial dialkyl carbamoyl chloride (31-78%). Similarly, *N*-alkylated 5-hydroxy INDY analogues **2a-h** were reacted with commercially available isocyanates providing *N*-monoalkyl carbamates **1d-n** in modest to high yields (12-95%).

To broaden the range of carbamoylated INDY derivatives **1**, we were also interested in more general synthetic methods, not limited to the use of marketed isocyanates or dialkyl carbamoyl chloride. We therefore moved to the preparation of an activated mixed carbonate by reacting *N*-alkylated 5-hydroxy

**Table 1** Inhibitory activities of 5-carbamoylated INDY derivatives **1a-q** against *eqBuChE/hAChE*View Article Online  
DOI: 10.1039/D3MD00706A

Entry	Compd	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	IC <sub>50</sub> (nM) ± SD <i>eqBuChE</i>	IC <sub>50</sub> (μM) ± SD <i>hAChE</i>
1	<b>1a</b>	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>	> 10 000	
2	<b>1b</b>	Ph	C <sub>2</sub> H <sub>5</sub>	CH <sub>3</sub>	68 ± 12	< 10% at 10 μM
3	<b>1c</b>	CH <sub>2</sub> Ph	C <sub>2</sub> H <sub>5</sub>	CH <sub>3</sub>	439 ± 52	< 10% at 10 μM
4	<b>1d</b>	CH <sub>3</sub>	<i>n</i> -Hept	H	1640 ± 160	
5	<b>1e</b>	Ph	<i>n</i> -Hept	H	154 ± 16	< 10% at 10 μM
6	<b>1f</b>	CH <sub>2</sub> Ph	<i>n</i> -Hept	H	26.7 ± 7.3	< 10% at 10 μM
7	<b>1g</b>	3-MePh	<i>n</i> -Hept	H	761 ± 35	
8	<b>1h</b>	3-ClPh	<i>n</i> -Hept	H	703 ± 40	
9	<b>1i</b>	4-ClPh	<i>n</i> -Hept	H	821 ± 60	
10	<b>1j</b>	2-naphthyl	<i>n</i> -Hept	H	> 10 000	
11	<b>1k</b>	3-MeOPh	<i>n</i> -Hept	H	2195 ± 296	
12	<b>1l</b>	Ph	<i>n</i> -Hex	H	470 ± 87	< 10% at 10 μM
13	<b>1m</b>	Ph	<i>n</i> -Bu	H	> 10 000	
14	<b>1n</b>	CH <sub>3</sub>	<i>c</i> -Hex	H	13 000 ± 3000	11 ± 3
15	<b>1o</b>	CH <sub>3</sub>	<i>n</i> -Hex	H	2000 ± 900	14 ± 2
16	<b>1p</b>	CH <sub>3</sub>	CH <sub>2</sub> -3-Py	H	12000 ± 3000	34 ± 7
17	<b>1q</b>	CH <sub>3</sub>	(CH <sub>2</sub> ) <sub>2</sub> Ph	H	6000 ± 3000	20 ± 3
18	<b>Tacrine</b>				1.9 ± 0.2	
19	<b>Donepezil</b>					(3.9 ± 0.7) · 10 <sup>-3</sup>

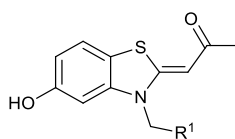
INDY analogue **2a** with carbonylation reagents such as 1,1'-carbonyldiimidazole (CDI) or *N,N'*-disuccinimidyl carbonate (DSC). In both cases, the resulting mixed carbonate proved to be highly unstable in solution, decomposing within a few hours. In the case of DSC, the unforeseen formation of the carbonate **7** helped us to overcome this issue, since, after optimizing the reaction conditions to prepare the carbonate **7** with a good yield (81%), the latter was successfully reacted with various amines to furnish the desired carbamoylated INDY derivatives **1o-q** (19-78%).

#### In vitro evaluation of synthesized 5-carbamoylated INDY derivatives **1a-q** on *eqBuChE/hAChE*

Inhibitory activities of the newly synthesized carbamoylated INDY derivatives **1a-q** were evaluated on *hAChE* and *eqBuChE* which shares a large amount of homology with *hBuChE*, using the method of Ellman.<sup>27</sup> The inhibitory activities are reported as IC<sub>50</sub> values (Table 1). Tacrine and Donepezil were used as reference standards (Table 1, Entries 18,19). We first focused on substituent variations at the carbamate moiety by comparing the performance of carbamoylated INDY derivatives **1a,d,n-q** (R<sup>1</sup> = Me) in inhibiting *eqBuChE* (Table 1, Entries 1,4

and 14-17). Among the first set of compounds, most of them exerted some inhibitory effects against *eqBuChE* with one- to two-digit micromolar IC<sub>50</sub> values ranging from 1.6 to 13 μM, except for compound **1a** which proved to be inactive towards *eqBuChE* (25% inhibition at 10 μM). As already mentioned in the literature with other carbamate-based cholinesterase inhibitors,<sup>10,28-32</sup> these results underline the impact of the size of the lipophilic alkyl chain at the carbamate moiety in inhibiting *BuChE*.

We then examined the influence of the *N*-alkyl appendage at the benzothiazole scaffold on the inhibitory activity of *eqBuChE*. A short set of *N*-benzylated INDY derivatives **1b,e,l,m** bearing different carbamates at C-5 were first evaluated (Table 1, Entries 2,5,12 and 13). To our delight, compounds **1b,1e** and **1l** showed two- to three-digit nanomolar IC<sub>50</sub> values (IC<sub>50</sub>= 68-470 nM), even though a much lower inhibitory activity was observed with compound **1m** (22% inhibition at 10 μM). Various substitutions at the *N*-benzyl appendage did not lead to a breakthrough (Table 1, entries 7-9 and 11), compounds **1g-i** displaying good but lower three-digit nanomolar potencies (IC<sub>50</sub>= 703-821 nM) while compound **1k** exhibited a low one-

**Table 2** Inhibitory activities of *N*-alkylated INDY analogues **2b-h** on *h*DYRK1A and *h*CLK1 kinasesView Article Online  
DOI: 10.1039/D3MD00700A

Entry	Compd	R <sup>1</sup>	IC <sub>50</sub> (nM) ± SD	
			<i>h</i> DYRK1A	<i>h</i> CLK1
1	<b>2a</b>	CH <sub>3</sub> (INDY)	240 <sup>a</sup>	44 <sup>b</sup>
2	<b>2b</b>	Ph	529 ± 40	54 ± 5
3	<b>2c</b>	CH <sub>2</sub> Ph	106 ± 27	17.0 ± 0.1
4	<b>2d</b>	3-MePh	429 ± 18	39 ± 12
5	<b>2e</b>	3-ClPh	885 ± 60	33 ± 1
6	<b>2f</b>	4-ClPh	nd <sup>c</sup> (75%)	40 ± 9
7	<b>2g</b>	2-naphtyl	nd <sup>c</sup> (24%)	nd <sup>c</sup> (72%)
8	<b>2h</b>	3-MeOPh	883 ± 81	76 ± 11

<sup>a</sup>from ref. [25]<sup>b</sup>from ref. [24]<sup>c</sup>nd: not determined (% inhibition at 10 μM)

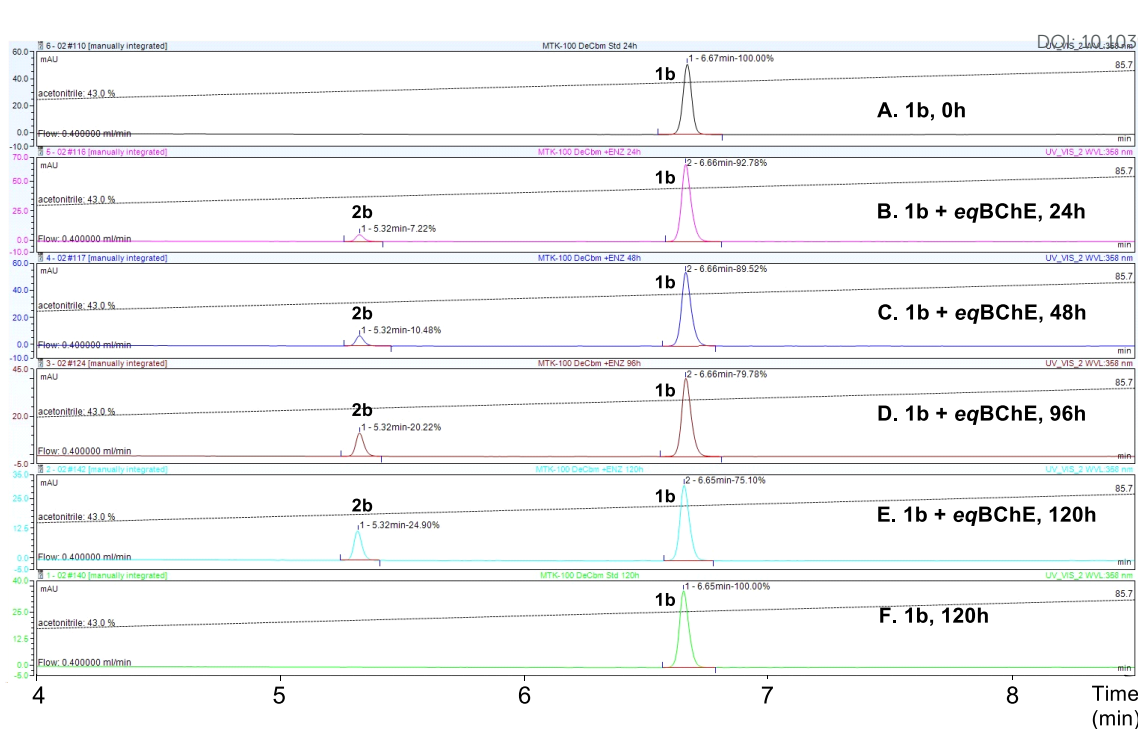
digit micromolar inhibitory activity (IC<sub>50</sub> = 2.2 μM). Switching the *N*-benzyl substituent to the more sterically hindered 2-naphtyl moiety in compound **1j** resulted in a significant drop in the affinity for *eq*BuChE (Table 1, entry 10).

In a last attempt to improve the performance of this new class of carbamate-based cholinesterase inhibitors, the inhibitory potency of compounds **1c** and **1f** bearing a more flexible pendant phenethyl at the nitrogen atom of the benzothiazole core were assessed against *eq*BuChE (Table 1, Entries 3 and 6). Whereas compound **1c** exhibited attractive inhibitory properties in a three-digit nanomolar range, we were pleased to note that compound **1f** proved to be the most potent *eq*BuChE inhibitor reaching the lowest two-digit IC<sub>50</sub> value (IC<sub>50</sub> = 27 nM).

We also looked briefly at the inhibitory potency of some carbamoylated INDY derivatives **1** towards *h*AChE. In short, among all the carbamoylated INDY derivatives **1b, 1c, 1e, 1f, 1i** and **1n-q**, only those bearing a *N*-ethyl pendant at the benzothiazole scaffold displayed two-digit micromolar inhibitory activities against *h*AChE in the same range as that observed towards *eq*BuChE (Table 1, Entries 14-17). More importantly, among the more potent carbamoylated INDY derivatives **1b, 1c, 1e, 1f** and **1i** against *eq*BuChE, namely those bearing a *N*-aromatic pendant, none showed apparent inhibitory properties towards *h*AChE, making them highly selective BuChE inhibitors (Table 1, entries 2, 3, 5, 6 and 12). These data strongly suggest that attachment of an aromatic moiety at the nitrogen of the benzothiazole core is a prerequisite to achieve highly potent and selective inhibitors towards BuChE *versus* AChE.

#### In vitro evaluation of *N*-alkylated INDY analogues on *h*DYRK1A and *h*CLK1 kinases

While most of the multitarget strategies reported in the literature<sup>33</sup> bring into play a single ligand that can interact in a concomitant manner with several targets, our approach deals with pseudo-irreversible carbamate inhibitors which are designed to interact first with cholinesterases while releasing putative *h*DYRK1A and *h*CLK1 inhibitors. According to this sequential dual inhibition mechanism, the aforementioned carbamoylated INDY derivatives **1a-q** are not expected to exhibit activity on *h*DYRK1A and *h*CLK1 kinases. For assays on *h*DYRK1A and *h*CLK1 kinases, we used staurosporine as a control inhibitor. As forecasted, a selection of carbamoylated INDY derivatives **1b, 1e** and **1i** showing good potency in BuChE inhibition were found to lack inhibitory activity against *h*DYRK1A and *h*CLK1 kinases (inhibition <10% at 10 μM). Lastly, the inhibitory activities of *N*-alkylated INDY analogues **2** were assessed against *h*DYRK1A/*h*CLK1 kinases with the ultimate goal of identifying a pair of compounds **1/2** endowed with sequential dual inhibitory activity against *eq*BuChE and *h*DYRK1A/*h*CLK1 kinases. As is clearly shown in Table 2, a substantial number of *N*-alkylated INDY analogues **2b-e** and **2h** efficiently inhibited both *h*DYRK1A/*h*CLK1 kinases in the same IC<sub>50</sub> values range as that observed for INDY, except for compounds **2f** and **2g**. Specifically, compounds **2b-e** and **2h** exhibited an activity towards *h*DYRK1A with three-digit nanomolar IC<sub>50</sub> values ranging from 106 to 885 nM while inhibiting *h*CLK1 with two-digit nanomolar IC<sub>50</sub> values ranging from 17 to 76 nM. It is worth noting that compound **2c** proved to be the most potent inhibitor against both *h*DYRK1A and *h*CLK1, even outperforming the inhibitory potency of INDY (Table 2, entries 1 and 3). These data are all the more interesting as the corresponding carbamoylated INDY derivatives **1c** and **1f** provide good to excellent inhibitory activities against *eq*BuChE (Table 1, entries



**Fig. 2** Decarbamylation HPLC study of **1b** by *eqBuChE*. A and F: **1b** in the absence of enzyme *eqBuChE*. B-E: decarbamylation reaction of **1b** in the presence of *eqBuChE*, forming the analogue of INDY **2b**.

3 and 6), making them appealing candidates as potent dual inhibitors with a good balance in inhibiting *eqBuChE* and *hDYRK1A/hCLK1* kinases.

### Stability, solubility and brain penetration

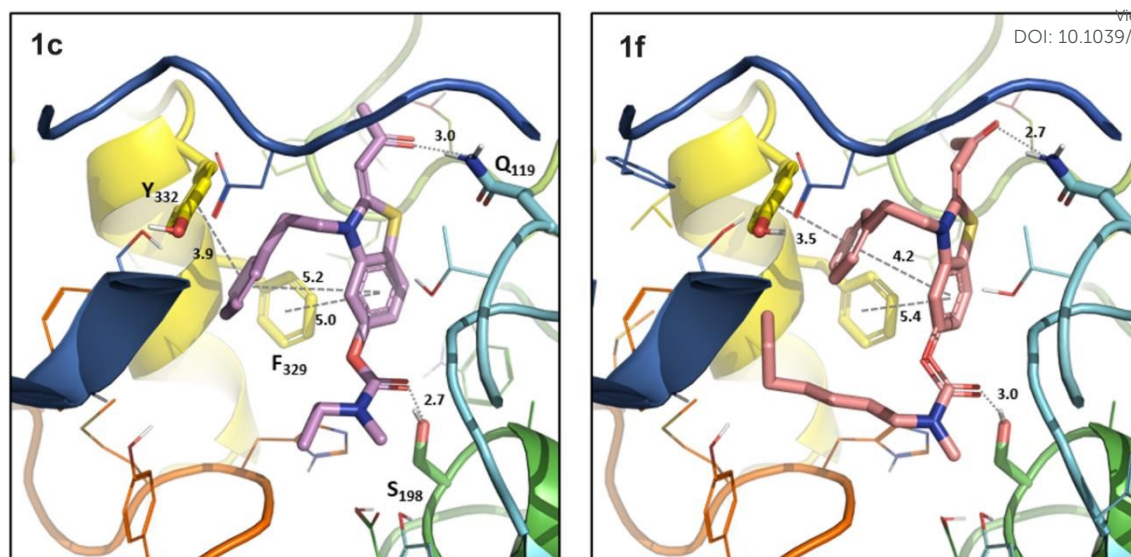
The ability of carbamoylated INDY derivatives **1b** and **1f** to cross the lipophilic Blood-Brain-Barrier (BBB) by passive diffusion was assessed. These two compounds were selected since they both exhibit excellent activities on *eqBuChE* and the corresponding decarbamoylated INDY analogues **2b** and **2c** showed good inhibition on *hDYRK1A/hCLK1* kinases. The lipophilicity of compounds **1b** and **1f** was first evaluated using the chromatographic hydrophobicity index (CHI) based on a reversed phase HPLC method at pH=7.4.<sup>34,35</sup> While moderate lipophilicity was measured for compound **1b** (CHI log D=3,0), a somewhat higher lipophilicity was recorded with compound **1f** (CHI log D=4,36). Simultaneously, we made use of the Parallel Artificial Membrane Permeability assay (PAMPA-BBB),<sup>36</sup> a convenient and low-cost method for the rapid assessment of passive BBB penetration by CNS-targeted drugs. Attempts to perform this physicochemical assay on compound **1f** were inconclusive, likely due to its too low solubility that prevents reproducible results. More generally speaking, these solubility issues were also observed with compounds **1d,1i** and **1o** which incorporate a long lipophilic carbamate chain. Pleasingly, compound **1b** proved to be highly permeable and an excellent candidate to cross the BBB (Log<sub>P<sub>e</sub></sub>=-4.70±0.14). The assay was performed using theophylline (a weak brain-penetrating compound Log<sub>P<sub>e</sub></sub>=-6.97±0.03) and corticosterone (a good brain-penetrating compound Log<sub>P<sub>e</sub></sub>=-4.81±0.02) as references. In

addition, compound **1b** was found to be soluble (28.2 μM in PBS at pH 7.4) and stable to hydrolysis, with no traces of decarbamoylated compound **2b** observed after several days.

### Decarbamylation study of **1b** by *eqBuChE*

The success of our multitarget approach is closely linked to the mechanism of *eqBuChE* inhibition by carbamate inhibitors **1**, which are assumed to promote carbamylation at the serine residue of the active site. To shed light on this pseudo-irreversible mechanism of action, we undertook to monitor *in vitro* decarbamylation of INDY derivative **1b** in the presence of *eqBuChE* by HPLC in a PBS buffer (pH 7.4). The reaction was conducted at 37°C with **1b** in the presence of *eqBuChE* for 5 days (Fig. 2, B-E) and compared with a control experiment in the absence of the enzyme (Fig. 2, A,F). Pleasingly, from aliquots taken daily, we were able to observe the formation of compound **2b** (7% after 24h and 25% after 5 days) while checking that decarbamylation of **1b** did not take place in the absence of *eqBuChE*. The moderate conversion observed is most likely due to the cumulative effects of the fairly concentrated solutions of **1b** required for UV detection together with slow regeneration of the carbamoylated *eqBuChE*. Nevertheless, this experiment clearly demonstrates that *eqBuChE* catalyzes decarbamylation of compound **1b** with concomitant release of INDY analogue **2b**. These observations highlight the pseudo-irreversible mechanism of this new class of carbamates while validating their ability to target *eqBuChE* and *hDYRK1A/hCLK1* kinases according to a sequential mechanism.





**Fig. 3** The representative positions of compound **1c** and **1f** in *eqBuChE* binding sites generated from the docking studies. The compound and the selected side chains of the binding site residues are in stick and the protein in ribbon representation. This figure was made with PYMOL (DeLano Scientific, 2002, San Carlo, USA).

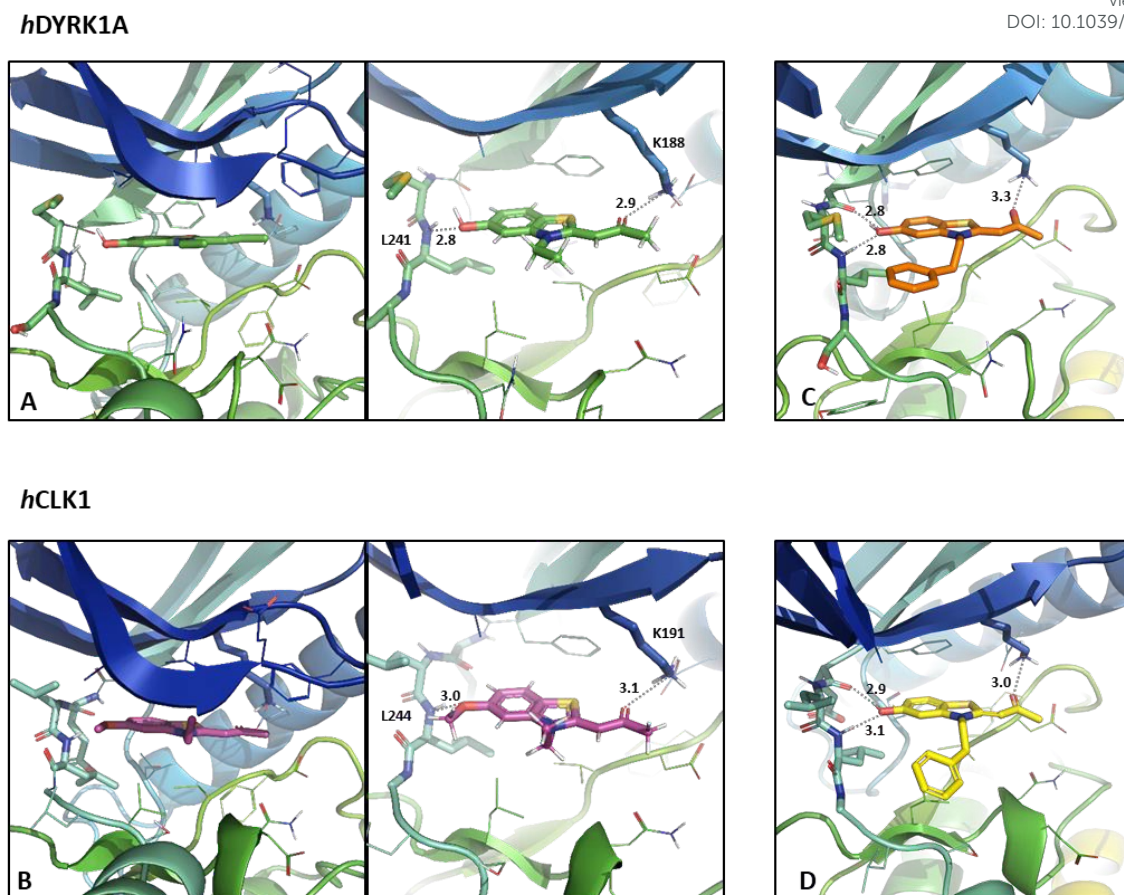
### Docking studies

**Cholinesterase binding: *eqBuChE*.** Firstly, in order to predict the ability of **1c** and **1f** to interact with the *eqBuChE* binding site, docking studies were performed using GOLD 2020.2.0 (Cambridge Crystallographic Data Center, CCDC) in a homology model of *eqBuChE*. By this docking, we wanted to check if the carbamate derivatives **1c** and specially **1f** with a long heptyl chain could access the *eqBuChE* catalytic triad (Ser198, Glu325, His438). During carbamylation the Ser198 residue plays the crucial role and according to the proposed carbamylation mechanism for rivastigmine by Bacalhau *et al.*,<sup>37</sup> the carbamate-to-serine grouping approach starts *via* a hydrogen bond between an oxygen atom of the ligand carbamate carbonyl and the hydroxyl group of Ser198. To ensure that in our model, a hydrogen bond constraint between the ligands' carbamate carbonyl and Ser198 hydroxyl group was applied during docking. At the same time the protonated Glu197 side chain (suggested by PropKa calculation) and the Ser198 were kept flexibles. As imposed by the constraint, in all generated poses the carbamate moiety was located near the catalytic triad.

The ChemPLP score fit values of generated poses of compound **1c** varied between 75.82-64.69 and **1f** between 89.41-73.08. The generated poses for the two compounds are very closed, they are both fixed in the binding site through two hydrogen bonds, one with Ser198 side chain (H bond constraint in docking) and second with Gln119 side chain (Fig. 3). The phenyl substituent adopts an orientation allowing it to established a  $\pi$ - $\pi$  stacking with Tyr332 residue of the peripheral anionic site. In conclusion, the both compounds are able to bind to the BuChE binding cavity and there is enough space to accommodate the long heptyl substituent of **1f** and the presence of phenyl ring in R<sup>1</sup> position brings a supplementary interaction in the BuChE through a  $\pi$ - $\pi$  stacking.

**Kinases binding: DYRK1A and CLK1.** In the second step of modelling studies, we sought to elucidate how decarbamoylated ligands can interact with the two kinases. To achieve that, we focused on the most potent ligand **2c**. The solved X-ray structure of INDY inhibitor co-crystallized with *hDYRK1A* (PDB ID: 3ANQ)<sup>25</sup> showed that the INDY aromatic ring is placed parallel to the beta sheet delimiting the ATP binding site and that the ligand binds through two hydrogen bonds: i) a first one with the Leu241-*hDYRK1A* backbone NH and ii) a second one with NH<sub>3</sub><sup>+</sup> of Lys188-*hDYRK1A* (Fig. 4A). Even though the lysine side chain is long and flexible, its orientation in the *hDYRK1A* binding site is stabilized by the salt bridges with Glu203-*hDYRK1A*. For *hCLK1*, an X-ray structure co-crystallized with INDY is not available, but a structure co-crystallized with a close INDY derivative inhibitor was available (PDB ID: 6YTE, K<sub>d</sub>=75 nM).<sup>38</sup> Visualization showed that the INDY derivative binds to the *hCLK1* in a very similar way (Fig. 4A, 4B). The ligand aromatic ring adopts the same orientation and it establishes similar hydrogen bonds to those in *hDYRK1A*: i) one with NH backbone of Leu244-*hCLK1* and ii) a second one with NH<sub>3</sub><sup>+</sup> of the Lys191-*hCLK1* side chain. The position of the lysine side chain in *hCLK1* is close to the one in *hDYRK1A*. Its position is also fixed by the salt bridges with neighboring Glu206-*hCLK1*. Therefore, the INDY binding in the two kinases is accomplished in the same way.

To gain further insight into the binding mode of compound **2c**, it was docked into *hDYRK1A* and *hCLK1* using the GOLD 2020.2.0 program applying the ChemPLP scoring function (Fig. 4C,D). The performance of our docking procedure was verified first by redocking the INDY in *hDYRK1A* and in *hCLK1*. GOLD positioned INDY correctly in the *hDYRK1A* cavity, however it systematically proposed two types of solutions, one observed in the X-ray structure with the hydroxyl group oriented towards Leu241 and the other with INDY turned over on itself where its hydroxyl group points towards Lys188. The same was observed during



**Fig. 4** Two perpendicular views of (A) INDY inhibitor position from the *hDYRK1A* X-ray structure and (B) INDY derivative position from the *hCLK1* X-ray structure compared to the compound **2c** positioned using the docking studies in (C) *hDYRK1A* binding site and (D) *hCLK1* binding site. The INDY, INDY derivative, compound **2c** and the selected residue or residue side chains of the binding site are in stick and the protein in ribbon representation. This figure was made with PYMOL (DeLano Scientific, 2002, San Carlo, USA).

docking in *hCLK1*. The possibility of these two orientations for benzothiazole derivatives was evoked already in the structural study by Rothweiler et al.<sup>39</sup> The INDY ChemPLP fit score was in the range of 62.56-52.73 for *hDYRK1A* and 66.14-57.80 in *hCLK1*. Afterwards, the docking studies for compound **2c** were performed and the results confirmed that **2c** reproduces the INDY binding well. For both kinases the poses proposed by GOLD converged to two ligand orientations: as for INDY, the best scoring ones were with the hydroxyl group positioned towards Leu241 and are represented on Fig. 4C,D. The compound **2c** ChemPLP fit score for the poses with the hydroxyl oriented toward Leu were between 70.71-69.31 in *hDYRK1A* and between 76.98-72.60 in *hCLK1*. In conclusion, compound **2c** is able to establish the two crucial hydrogen bonds with both kinases, the first one with the Leu241 backbone NH and the second one with NH<sub>3</sub><sup>+</sup> of Lys188 in the same way as INDY. However, the docking poses do not give us an idea of the particular role played by the phenyl substituent in the kinase binding.

## Conclusions

Although a few rare treatments are available to improve cognitive dysfunction in Alzheimer's patients, there is no cure despite numerous dedicated research programs, which remains a major unmet therapeutic need. To achieve this goal, the development of multitarget anti-Alzheimer agents has been one of the main lines of research deployed in recent years. In this context, we sought to develop a series of novel rivastigmine-INDY hybrids **1** targeting both cholinesterases and DYRK1A/CLK1 kinases, identified as relevant targets against AD. To this end, a panel of hybrids **1a-q** were easily prepared in a few synthetic steps from commercial products *via* carbamoylation of various INDY derivatives **2a-h**.

Most rivastigmine-INDY hybrids bearing *N*-aromatic substituents demonstrated good and selective *eqBuChE* inhibitory activities ranging from two- to three-digit nanomolar IC<sub>50</sub> values, whereas those equipped with an *N*-ethyl substituent exhibited in most cases much lower *eqBuChE* inhibitory activities in the range of one- to two-digit micromolar IC<sub>50</sub> values and without *eqBuChE* selectivity *versus hAChE*. A docking study performed on *eqBuChE* with compounds **1c** and **1f** provided further insight into the role of the aromatic group that is involved in a  $\pi$ - $\pi$  stacking with Tyr332 in the peripheral anionic site while placing the carbamate moiety in close

proximity to the active site serine residue. We were thus able to confirm the pseudo-irreversible mode of action of these new carbamoylated INDY derivatives **1** by HPLC monitoring of the decarbamoylation of compound **1b** in the presence of *eq*BuChE. Confirmation of their covalent mode of action towards *eq*BuChE paved the way for their development as multitarget ligands *via* a sequential-type mechanism on cholinesterases and DYRK1A/CLK1 kinases. To our great satisfaction, most decarbamoylated compounds **2b-e** and **2h** in turn revealed strong inhibitory potency towards *h*DYRK1A and *h*CLK1 kinases ( $IC_{50} = 106-885$  nM and  $IC_{50} = 17-76$  nM, respectively). As expected, a molecular docking study confirmed that the INDY analogue **2c** adopts the same binding mode as that observed between INDY and both DYRK1A/CLK1 kinases. Interestingly, while the molecular docking study of the carbamoylated INDY derivatives **1c** and **1f** against *eq*BuChE highlighted the crucial role played by the *N*-aromatic pendant group in positioning properly the carbamate moiety in the enzyme's active site, this structural element appears as a mere bystander in the binding mode of INDY analogues **2c** into the ATP pocket of DYRK1A and CLK1 kinases.

Overall, these SAR and molecular docking studies provided the main key structural features for designing rivastigmine-INDY hybrids **1** with dual activity, through a sequential mechanism, towards *eq*BuChE and DYRK1A/CLK1 kinases. Finally, identification of the rivastigmine-INDY hybrid **1b** giving rise to well-balanced potencies against *eq*BuChE and DYRK1A/CLK1 kinases along with a good ADMET profile gives the green light to consider further biological developments.

## Experimental section

### Materials and methods

**Chemistry.** All commercial reagents were used without further purification. The solvents were dried with appropriate desiccants and distilled prior to use or were obtained anhydrous from commercial suppliers. Silica gel (60, 230-400 mesh or 70-230 mesh) was used for column chromatography. Reactions were monitored by thin layer chromatography on silica gel precoated aluminum plates. UV light at 254 nm or  $KMnO_4$  stains were used to visualize TLC plates.  $^1H$ ,  $^{13}C$  NMR spectra were recorded using a spectrometer operating at 300 and 75 MHz respectively. Abbreviations used for peak multiplicities are s: singlet, d: doublet, t: triplet, q: quadruplet dd = doublet of doublet, br = broad and m: multiplet. Coupling constants *J* are in Hz and chemical shifts are given in ppm and calibrated with  $DMSO-d_6$  or  $CDCl_3$  (residual solvent signals).  $^1H$  NMR spectra obtained in  $CDCl_3$  were referenced to 7.26 ppm.  $^{13}C$  NMR spectra obtained in  $CDCl_3$  were referenced to 77.16 ppm and in  $DMSO-d_6$  were referenced to 39.52 ppm. Compounds **2a** (INDY), **3a** and **4a** (TG003) were prepared according to the procedure described in the literature<sup>24,25</sup> starting from commercially available 5-methoxy-2-methylbenzothiazole (Fisher Scientific). Commercial 2-methylbenzothiazol-5-ol was purchased from Fisher Scientific.

### Synthetic procedures

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DOI: 10.1039/D3MD00708A

#### General procedure A for the synthesis of compounds **1a-c**

The corresponding compound **2a-c** and potassium carbonate were suspended in dry dimethylformamide. *N,N'*-dialkylcarbamoyl chloride was added and the mixture was stirred at 20°C for 18 h. The reaction was hydrolyzed with a saturated solution of ammonium chloride (40 mL). The aqueous phase was extracted three times with dichloromethane (15 mL). The organic phases were washed with brine, dried on  $MgSO_4$  and evaporated to dryness. The crude product was purified by chromatography to afford compounds **1a-c**.

*(Z)*-3-Ethyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl dimethylcarbamate (**1a**). According to the general procedure A, from INDY **2a** (50 mg, 0.21 mmol) and potassium carbonate (44.1 mg, 0.32 mmol) in dry DMF (5 mL). *N,N'*-dimethylcarbamoyl chloride (29  $\mu$ L, 0.32 mmol) was added. Flash chromatography on silica gel (EtOAc) yielded compound **1a** (51 mg, 78%) as white crystals. m.p. 176-177°C;  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  7.47 (d, *J* = 8.3 Hz, 1H), 6.92-6.85 (m, 2H), 5.85 (s, 1H), 3.98 (q, *J* = 7.2 Hz, 2H), 3.09 (s, 3H), 3.00 (s, 3H), 2.21 (s, 3H), 1.33 (t, *J* = 7.2 Hz, 3H);  $^{13}C$  NMR (75 MHz,  $CDCl_3$ )  $\delta$  191.27, 160.55, 154.70, 150.75, 139.87, 123.78, 122.48, 116.37, 103.89, 90.25, 40.73, 36.80, 36.53, 29.03, 11.59; IR (neat, v): 2932, 1724, 1472, 1382, 1183; HRMS (ESI): *m/z* calcd. for  $C_{15}H_{19}N_2O_3S$ : 307.1111 [M+H]<sup>+</sup>, found 307.1119.

*(Z)*-3-Benzyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl ethyl(methyl) carbamate (**1b**). According to the general procedure A, from INDY **2b** (70 mg, 0.24 mmol) and potassium carbonate (49 mg, 0.35 mmol) in dry DMF (2 mL). *N*-ethyl-*N*-methyl-carbamoyl chloride (29  $\mu$ L, 0.32 mmol) was added. Flash chromatography on silica gel (EtOAc) yielded compound **1b** (65 mg, 72%) as a beige solid. m.p. 133-134°C;  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  7.54 (d, *J* = 8.4 Hz, 1H), 7.41-7.28 (m, 3H), 7.15 (dt, *J* = 7.7, 1.1 Hz, 2H), 7.00-6.90 (m, 1H), 6.87 (s, 1H), 5.85 (s, 1H), 5.18 (s, 2H), 3.41 (m, 1+1H, rotamer 1+2), 3.03 (s, 1.5H, rotamer 1), 2.97 (s, 1.5H, rotamer 2), 2.16 (s, 3H), 1.19 (m, 1.5+1.5H, rotamer 1+2);  $^{13}C$  NMR (75 MHz,  $CDCl_3$ )  $\delta$  191.73, 161.26, 150.91, 140.66, 133.94, 129.23, 128.09, 126.20, 123.59, 122.59, 116.86, 104.28, 91.40, 49.37, 44.23 (rotamer 1), 44.16 (rotamer 2), 34.36 (rotamer 1), 33.91 (rotamer 2), 29.13, 13.31 (rotamer 1), 12.51 (rotamer 2); HRMS (ESI): *m/z* calcd. for  $C_{21}H_{23}N_2O_3S$ : 383.1429 [M+H]<sup>+</sup>, found 383.1429.

*(Z)*-3-Phenethyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl ethyl(methyl) carbamate (**1c**). According to the general procedure A, from INDY **2c** (100 mg, 0.32 mmol) and potassium carbonate (44 mg, 0.32 mmol) in dry DMF (1.5 mL). *N*-ethyl-*N*-methyl-carbamoyl chloride (106  $\mu$ L, 0.96 mmol) was added. Purification by preparative TLC (Et<sub>2</sub>O/ ethylacetate 9:1) afforded **1c** (39 mg, 31%) as a beige solid. m.p. 197-198°C;  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  7.54 (d, *J* = 8.4 Hz, 1H), 7.41-7.28 (m, 3H), 7.15 (dt, *J* = 7.7, 1.1 Hz, 2H), 7.00-6.90 (m, 1H), 6.87 (s, 1H), 5.85 (s, 1H), 5.18 (s, 2H), 3.41 (m, 1+1H, rotamer 1+2), 3.03 (s, 1.5H, rotamer 1), 2.97 (s, 1.5H, rotamer 2), 2.16 (s, 3H), 1.19 (m,

1.5+1.5H, rotamer 1+2);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.73, 161.26, 150.91, 140.66, 133.94, 129.23, 128.09, 126.20, 123.59, 122.59, 116.86, 104.28, 91.40, 49.37, 44.23 (rotamer 1), 44.16 (rotamer 2), 34.36 (rotamer 1), 33.91 (rotamer 2), 29.13, 13.31 (rotamer 1), 12.51 (rotamer 2); HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{22}\text{H}_{25}\text{N}_2\text{O}_3\text{S}$ : 397.1586  $[\text{M}+\text{H}]^+$ , found 397.1590.

#### General procedure B for the synthesis of compounds **1d-n**

In a tube, to a suspension of **2a-h** in dichloromethane or dry DMF were successively added the alkyl isocyanate and triethylamine. The tube was sealed and the resulting mixture was stirred at 20–65 °C for 18 hours. Thereafter, the solvent was removed under vacuum and the residue was dissolved in a minimum amount of dichloromethane. A mixture of diethyl ether and pentane was gradually added until precipitation. Filtration of the solid gave compounds **1d-n**.

*(Z)*-3-Ethyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl heptylcarbamate (**1d**). According to the general procedure B, from **2a** (200 mg, 0.85 mmol) in dichloromethane (5 mL). *n*-Heptyl isocyanate (205  $\mu\text{L}$ , 1.27 mmol) and triethylamine (12  $\mu\text{L}$ , 85  $\mu\text{mol}$ ) were added to give **1d** (302 mg, 94%) as a beige solid. m.p. 159–160 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.48 (d,  $J$  = 8.3 Hz, 1H), 7.00–6.81 (m, 2H), 5.87 (s, 1H), 5.18 (t,  $J$  = 5.8 Hz, 1H), 4.00 (q,  $J$  = 7.2 Hz, 2H), 3.27 (q,  $J$  = 6.7 Hz, 2H), 2.23 (s, 3H), 1.57 (d,  $J$  = 8.4 Hz, 2H), 1.39–1.26 (m, 10H), 0.98–0.78 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.43, 160.68, 154.50, 150.44, 139.99, 123.93, 122.63, 116.25, 103.77, 90.36, 41.50, 40.84, 31.85, 29.93, 29.13, 29.04, 26.84, 22.70, 14.18, 11.68; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{20}\text{H}_{29}\text{N}_2\text{O}_3\text{S}$ : 377.1899  $[\text{M}+\text{H}]^+$ , found 377.1901.

*(Z)*-3-Benzyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl heptylcarbamate (**1e**). According to the general procedure B, from **2b** (100 mg, 0.34 mmol) in dichloromethane (3.4 mL). *n*-Heptyl isocyanate (81  $\mu\text{L}$ , 0.5 mmol) and triethylamine (9.4  $\mu\text{L}$ , 67  $\mu\text{mol}$ ) were added to give **1e** (91.0 mg, 62%) as a pale pink solid.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.54 (d,  $J$  = 8.4 Hz, 1H), 7.41–7.29 (m, 3H), 7.21–7.11 (m, 2H), 6.94 (dd,  $J$  = 8.4, 2.1 Hz, 1H), 6.88 (d,  $J$  = 2.1 Hz, 1H), 5.86 (s, 1H), 5.17 (s, 2H), 5.00 (s, 1H), 3.24 (q,  $J$  = 6.7 Hz, 2H), 2.16 (s, 3H), 1.56 (d,  $J$  = 7.3 Hz, 2H), 1.29 (d,  $J$  = 9.1 Hz, 8H), 0.94–0.80 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.79, 161.29, 154.39, 150.47, 140.58, 133.85, 129.20, 128.09, 126.18, 123.49, 122.55, 116.66, 104.17, 91.36, 49.34, 41.39, 31.77, 29.80, 29.09, 28.97, 26.76, 22.63, 14.13; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{25}\text{H}_{31}\text{N}_2\text{O}_3\text{S}$ : 439.2055  $[\text{M}+\text{H}]^+$ , found 439.2047.

*(Z)*-2-(2-Oxopropylidene)-3-phenethyl-2,3-dihydrobenzothiazol-5-yl heptylcarbamate (**1f**). According to the general procedure B, from **2c** (100 mg, 0.32 mmol) in DMF (1.5 mL). *n*-Heptyl isocyanate (155  $\mu\text{L}$ , 0.96 mmol) and triethylamine (9  $\mu\text{L}$ , 65  $\mu\text{mol}$ ) were added to give **1f** (138 mg, 95%) as a beige solid. m.p. 141–142 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.48 (d,  $J$  = 8.4 Hz, 1H), 7.40–7.15 (m, 5H), 6.91 (dd,  $J$  = 8.4, 2.0 Hz, 1H), 6.84 (d,  $J$  = 2.0 Hz, 1H), 5.83 (s, 1H), 5.12 (t,  $J$  = 5.8 Hz, 1H), 4.15 (t,  $J$  = 7.8 Hz, 2H), 3.28 (q,  $J$  = 6.7 Hz, 2H), 3.04 (t,  $J$  = 7.7 Hz, 2H), 2.22 (s, 3H), 1.60 (t,  $J$  = 7.1 Hz, 2H), 1.45–1.18 (m, 8H), 0.90 (q,  $J$  = 6.6, 4.7 Hz,

3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.52, 160.88, 154.44, 150.39, 140.13, 137.38, 129.03, 128.84, 127.26, 123.72, 122.59, 116.42, 103.89, 90.75, 47.42, 41.51, 32.76, 31.86, 29.94, 29.15, 29.06, 26.85, 22.71, 14.19; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{26}\text{H}_{33}\text{N}_2\text{O}_3\text{S}$ : 453.2212  $[\text{M}+\text{H}]^+$ , found 453.2211.

*(Z)*-3-(3-Methylbenzyl)-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl heptylcarbamate (**1g**). According to the general procedure B, from **2d** (70 mg, 0.22 mmol) in dry DMF (1.0 mL). *n*-Heptyl isocyanate (91  $\mu\text{L}$ , 0.56 mmol) and triethylamine (6.3  $\mu\text{L}$ , 45  $\mu\text{mol}$ ) were added to give **1g** (72 mg, 71%) as a beige solid. m.p. 169–170 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.51 (d,  $J$  = 8.4 Hz, 1H), 7.21 (d,  $J$  = 8.0 Hz, 1H), 7.10 (d,  $J$  = 7.6 Hz, 1H), 6.97–6.89 (m, 3H), 6.87 (d,  $J$  = 2.0 Hz, 1H), 5.85 (s, 1H), 5.14 (d,  $J$  = 11.1 Hz, 3H), 3.23 (td,  $J$  = 7.2, 6.0 Hz, 2H), 2.31 (s, 3H), 2.16 (s, 3H), 1.59–1.50 (m, 2H), 1.32–1.26 (m, 8H), 0.91–0.86 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.84, 161.39, 154.40, 150.50, 140.73, 139.12, 133.87, 129.14, 128.93, 126.73, 123.64, 123.26, 122.60, 116.64, 104.20, 91.42, 49.47, 41.45, 31.82, 29.87, 29.17, 29.02, 26.81, 22.70, 21.58, 14.16; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{26}\text{H}_{33}\text{N}_2\text{O}_3\text{S}$ : 453.2212  $[\text{M}+\text{H}]^+$ , found 453.2225.

*(Z)*-3-(3-Chlorobenzyl)-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl heptylcarbamate (**1h**). According to the general procedure B, from **2e** (100 mg, 0.31 mmol) in dry DMF (2.0 mL). *n*-Heptyl isocyanate (73  $\mu\text{L}$ , 0.45 mmol) and triethylamine (8.5  $\mu\text{L}$ , 61  $\mu\text{mol}$ ) were added to give **1h** as an off-white solid. Purification by TLC on  $\text{SiO}_2$  (ethyl acetate/pentane 2:3) afforded **1h** (17 mg, 12%). m.p. 169–170 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.53 (d,  $J$  = 8.4 Hz, 1H), 7.27 (dd,  $J$  = 4.7, 1.5 Hz, 2H), 7.15 (q,  $J$  = 1.4 Hz, 1H), 7.03–6.91 (m, 2H), 6.85 (d,  $J$  = 2.0 Hz, 1H), 5.81 (s, 1H), 5.13 (s, 2H), 5.08 (t,  $J$  = 5.8 Hz, 1H), 3.32–3.16 (m, 2H), 2.17 (s, 3H), 1.55 (m, 2H), 1.31–1.23 (m, 8H), 0.88 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.99, 161.24, 154.35, 150.57, 140.40, 136.05, 135.30, 130.66, 128.52, 126.40, 124.33, 123.63, 122.81, 116.84, 104.00, 91.47, 48.90, 41.49, 31.84, 29.89, 29.21, 29.03, 26.82, 22.70, 14.18; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{25}\text{H}_{30}\text{N}_2\text{O}_3\text{S}^{35}\text{Cl}$ : 473.1666  $[\text{M}+\text{H}]^+$ , found 473.1655.

*(Z)*-3-(4-Chlorobenzyl)-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl heptylcarbamate (**1i**). According to the general procedure B, from **2f** (100 mg, 0.30 mmol) in dry DMF (2.0 mL). *n*-Heptyl isocyanate (64  $\mu\text{L}$ , 0.45 mmol) and triethylamine (8.5  $\mu\text{L}$ , 61  $\mu\text{mol}$ ) were added to give **1i** (88 mg, 62%) as an off white solid. m.p. 188–189 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.52 (d,  $J$  = 8.4 Hz, 1H), 7.37–7.28 (m, 2H), 7.08 (d,  $J$  = 8.5 Hz, 2H), 6.94 (dd,  $J$  = 8.4, 2.0 Hz, 1H), 6.85 (d,  $J$  = 2.0 Hz, 1H), 5.81 (s, 1H), 5.12–5.08 (m, 3H), 3.31–3.18 (m, 2H), 2.16 (s, 3H), 1.54 (d,  $J$  = 7.1 Hz, 2H), 1.38–1.20 (m, 8H), 0.96–0.80 (m, 3H)-contains traces of diethylether;  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.91, 161.18, 154.34, 150.52, 140.40, 134.07, 132.40, 129.50, 127.66, 123.61, 122.77, 116.77, 104.03, 91.42, 48.80, 41.46, 31.83, 29.88, 29.19, 29.02, 26.81, 22.69, 14.19; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{25}\text{H}_{30}\text{N}_2\text{O}_3\text{S}^{35}\text{Cl}$ : 473.1666  $[\text{M}+\text{H}]^+$ , found 473.1653.

*(Z)*-3-(2-Naphtyl)-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl heptylcarbamate (**1j**). According to

the general procedure B, from **2g** (62 mg, 0.18 mmol) in dry DMF (1.0 mL). *n*-Heptyl isocyanate (43  $\mu$ L, 0.27 mmol) and triethylamine (5  $\mu$ L, 0.036 mmol) were added to give **1j** (87 mg, 77%) as an off white solid. m.p. 168-169 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.84 (m, 2H), 7.75 (dd,  $J = 6.1, 3.4$  Hz, 1H), 7.60-7.44 (m, 4H), 7.30 (dd,  $J = 8.5, 1.9$  Hz, 1H), 6.95 (dd,  $J = 8.4, 2.0$  Hz, 1H), 6.91 (d,  $J = 2.0$  Hz, 1H), 5.90 (s, 1H), 5.32 (s, 2H), 5.01 (s, 1H), 3.21 (td,  $J = 7.2, 6.0$  Hz, 2H), 2.14 (s, 3H), 1.52 (d,  $J = 7.4$  Hz, 2H), 1.36-1.21 (m, 8H), 0.92-0.83 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.90, 161.42, 154.37, 150.53, 140.62, 133.37, 132.97, 131.21, 129.25, 127.90, 127.81, 126.72, 126.41, 124.84, 123.91, 123.55, 122.61, 116.72, 104.23, 91.47, 49.61, 41.39, 31.77, 29.78, 29.07, 28.96, 26.75, 22.63, 14.13; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{29}\text{H}_{33}\text{N}_2\text{O}_3\text{S}$ : 489.2212  $[\text{M}+\text{H}]^+$ , found 489.2199.

(*Z*)-3-(3-Methoxybenzyl)-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl heptylcarbamate (**1k**). According to the general procedure B, from **2h** (100 mg, 0.31 mmol) in dry DMF (2.0 mL). *n*-Heptyl isocyanate (74  $\mu$ L, 0.46 mmol) and triethylamine (8.5  $\mu$ L, 61  $\mu$ mol) were added to give **1k** (121 mg, 85%) as an off white solid. m.p. 156-157 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.52 (d,  $J = 8.3$  Hz, 1H), 7.25 (t,  $J = 7.9$  Hz, 2H), 6.93 (dd,  $J = 8.4, 2.1$  Hz, 1H), 6.89-6.79 (m, 2H), 6.75-6.65 (m, 2H), 5.86 (s, 1H), 5.13 (s, 2H), 5.08 (d,  $J = 5.4$  Hz, 1H), 3.76 (s, 3H), 3.23 (q,  $J = 6.7$  Hz, 2H), 2.16 (s, 3H), 1.55 (t,  $J = 7.3$  Hz, 2H), 1.37-1.23 (m, 8H), 0.93-0.82 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.86, 161.36, 160.30, 154.38, 150.47, 140.66, 135.59, 130.42, 123.67, 122.66, 118.39, 116.66, 113.07, 112.28, 104.18, 91.42, 55.39, 49.39, 41.46, 31.84, 29.89, 29.21, 29.03, 26.82, 22.70, 14.19; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{26}\text{H}_{33}\text{N}_2\text{O}_4\text{S}$ : 469.2161  $[\text{M}+\text{H}]^+$ , found 469.2157.

(*Z*)-3-Benzyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl hexylcarbamate (**1l**). According to the general procedure B, from **2b** (100 mg, 0.34 mmol) in dichloromethane (2.0 mL). *n*-Hexyl isocyanate (74  $\mu$ L, 0.5 mmol) and triethylamine (5  $\mu$ L, 35  $\mu$ mol) were added to give **1l** (90 mg, 63%) as an off white solid. m.p. 185-186 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.52 (d,  $J = 8.4$  Hz, 1H), 7.40-7.28 (m, 3H), 7.21-7.08 (m, 2H), 6.94 (dd,  $J = 8.4, 2.1$  Hz, 1H), 6.88 (d,  $J = 2.0$  Hz, 1H), 5.85 (s, 1H), 5.16 (s, 2H), 5.10 (d,  $J = 6.3$  Hz, 1H), 3.30-3.17 (m, 2H), 2.16 (s, 3H), 1.62-1.47 (m, 2H), 1.37-1.24 (m, 6H), 0.96-0.81 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.87, 161.36, 154.39, 150.48, 140.66, 133.92, 129.28, 128.16, 126.24, 123.67, 122.67, 116.68, 104.18, 91.43, 49.43, 41.45, 31.53, 29.84, 29.17, 26.51, 22.66, 14.13; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{24}\text{H}_{29}\text{N}_2\text{O}_3\text{S}$ : 425.1899  $[\text{M}+\text{H}]^+$ , found 425.1888.

(*Z*)-3-Benzyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl butylcarbamate (**1m**). According to the general procedure B, from **2b** (100 mg, 0.34 mmol) in DMF (2.0 mL). *n*-Butyl isocyanate (94  $\mu$ L, 0.84 mmol) and triethylamine (14  $\mu$ L, 0.1 mmol) were added and stirred at 20 °C to give **1m** (110 mg, 82%) as a grey solid. m.p. 197-198 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.53 (d,  $J = 8.4$  Hz, 1H), 7.42-7.27 (m, 3H), 7.23-7.09 (m, 2H), 6.94 (dd,  $J = 8.4, 2.0$  Hz, 1H), 6.88 (d,  $J = 2.0$  Hz, 1H), 5.86 (s, 1H), 5.17 (s, 2H), 5.01 (s, 1H), 3.38-3.13 (m, 2H), 2.16 (s, 3H), 1.61-1.47 (m, 2H), 1.47-1.27 (m, 2H), 0.94 (t,  $J = 7.3$  Hz, 3H);  $^{13}\text{C}$  NMR (75 MHz,

$\text{CDCl}_3$ )  $\delta$  191.90, 161.36, 154.40, 150.45, 140.68, 133.93, 129.30, 128.18, 126.26, 123.73, 122.71, 116.68, 104.18, 91.45, 49.45, 41.15, 31.95, 29.21, 20.02, 13.85; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{22}\text{H}_{25}\text{N}_2\text{O}_3\text{S}$ : 397.1586  $[\text{M}+\text{H}]^+$ , found 397.1576.

(*Z*)-3-Ethyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl cyclohexylcarbamate (**1n**). According to the general procedure B, from **2a** (50 mg, 0.21 mmol) in DMF (2 mL). Cyclohexyl isocyanate (45  $\mu$ L, 0.35 mmol) and triethylamine (44  $\mu$ L, 0.32 mmol) were added and stirred at 20 °C to give **1n**. Purification by flash chromatography on silica gel (EtOAc/PE 1:2) gave compound **1n** (43 mg, 56%) as a white solid. m.p. 160-162 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.50 (d,  $J = 8.3$  Hz, 1H), 6.97 (s, 1H), 6.92 (d,  $J = 8.2$  Hz, 1H), 5.88 (s, 1H), 4.95 (d,  $J = 8.5$  Hz, 1H), 4.02 (q,  $J = 7.2$  Hz, 2H), 3.59 (br s, 1H), 2.25 (s, 3H), 2.09-1.97 (m, 2H), 1.83-1.69 (m, 2H), 1.70-1.53 (m, 2H), 1.37 (t,  $J = 7.2$  Hz, 3H), 1.31-1.15 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.29, 160.54, 153.56, 150.36, 139.80, 123.57, 122.40, 116.22, 103.77, 90.24, 50.31, 40.72, 33.20, 29.01, 25.42, 24.82, 11.56; IR (neat,  $\nu$ ): 3291, 2940, 1711, 1471, 1446, 1188, 1007; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{19}\text{H}_{25}\text{N}_2\text{O}_3\text{S}$ : 361.1581  $[\text{M}+\text{H}]^+$ , found 361.1589.

#### General procedure C for the synthesis of compounds 1o-q.

To a solution of **7** in dry dichloromethane (2.0 mL) were respectively added the corresponding amine and triethylamine. The reaction was stirred at 20 °C. Thereafter, water (10 mL) was added, and extracted three times with dichloromethane. The organic layers were washed with brine, dried on  $\text{MgSO}_4$  and evaporated to dryness. The resulting solid was purified by flash chromatography on silica gel.

(*Z*)-3-Ethyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl hexylcarbamate (**1o**). The title compound **1o** was prepared according to the general procedure C with compound **7** (150 mg, 0.251 mmol; containing 17%-mol **2a**), *n*-hexylamine (36  $\mu$ L, 0.28 mmol) and triethylamine (42  $\mu$ L, 0.3 mmol). The reaction was stirred for 4.5h. Flash chromatography (EtOAc/ $\text{CH}_2\text{Cl}_2$  1:9) yielded **1o** (71 mg, 78%) as a white solid. m.p. 172-174 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.47 (d,  $J = 8.3$  Hz, 1H), 7.00-6.84 (m, 2H), 5.86 (s, 1H), 5.28 (dd,  $J = 8.1, 4.0$  Hz, 1H), 3.99 (q,  $J = 7.2$  Hz, 2H), 3.26 (q,  $J = 6.8$  Hz, 2H), 2.23 (s, 3H), 1.63-1.51 (m, 2H), 1.33 (q,  $J = 5.7, 4.2$  Hz, 9H), 0.94-0.83 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.41, 160.69, 154.51, 150.42, 139.94, 123.86, 122.60, 116.27, 103.80, 90.34, 41.47, 40.82, 31.55, 29.85, 29.09, 26.53, 22.66, 14.13, 11.66; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{19}\text{H}_{27}\text{N}_2\text{O}_3\text{S}$ : 363.1742  $[\text{M}+\text{H}]^+$ , found 363.1730.

(*Z*)-3-Ethyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl (pyridin-3-ylmethyl) carbamate (**1p**). The title compound **1p** was prepared according to the general procedure C with compound **7** (162 mg, 0.268 mmol; containing 18%-mol **2a**), 3-picolylamine (28 mg, 0.255 mmol) and triethylamine (43  $\mu$ L, 0.31 mmol). The reaction was stirred for 2h. Flash chromatography (EtOAc) on silica gel yielded **1p** (36 mg, 38%) as a beige solid. m.p. 170-171 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.62 (d,  $J = 21.2$  Hz, 2H), 7.75 (d,  $J = 8.1$  Hz, 1H), 7.52 (d,  $J = 8.2$  Hz, 1H), 7.41-7.29 (m, 1H), 6.93 (d,  $J = 10.6$  Hz, 2H), 5.89 (d,  $J = 2.0$  Hz, 1H), 5.61 (s, 1H), 4.51 (d,

$J = 6.2$  Hz, 2H), 4.02 (q,  $J = 7.3$  Hz, 2H), 2.24 (s, 3H), 1.37 (t,  $J = 7.3$  Hz, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.49, 160.63, 154.87, 150.24, 149.12, 148.94, 139.90, 135.79, 133.93, 123.97, 123.72, 122.58, 116.24, 103.78, 90.38, 42.84, 40.81, 29.03, 11.62; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{19}\text{H}_{20}\text{N}_3\text{O}_3\text{S}$ : 370.1225  $[\text{M}+\text{H}]^+$ , found 370.1219.

*(Z)*-3-Ethyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl phenethylcarbamate (**1q**). The title compound **1q** was prepared according to the general procedure C with compound **7** (150 mg, 0.251 mmol; containing 17%-mol **2a**), phenethylamine (35  $\mu\text{L}$ , 0.28 mmol) and triethylamine (42  $\mu\text{L}$ , 0.3 mmol). The reaction was stirred for 3h. Flash chromatography on silica gel ( $\text{EtOAc}/\text{CH}_2\text{Cl}_2$  1:9) and trituration ( $\text{CH}_2\text{Cl}_2$ , pentane) yielded **1q** (23 mg, 19%) as a white solid. m.p. 151-153 °C;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.48 (d,  $J = 8.2$  Hz, 1H), 7.38-7.19 (m, 5H), 6.97-6.78 (m, 2H), 5.87 (s, 1H), 5.29 (t,  $J = 6.1$  Hz, 1H), 4.00 (q,  $J = 7.2$  Hz, 2H), 3.56 (q,  $J = 6.7$  Hz, 2H), 2.91 (t,  $J = 7.0$  Hz, 2H), 2.24 (s, 3H), 1.35 (t,  $J = 7.2$  Hz, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  191.43, 160.67, 154.47, 150.31, 139.95, 138.53, 128.91, 128.83, 126.78, 123.98, 122.64, 116.25, 103.77, 90.37, 42.49, 40.83, 36.01, 29.11, 11.68; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{21}\text{H}_{23}\text{N}_2\text{O}_3\text{S}$ : 383.1429  $[\text{M}+\text{H}]^+$ , found 383.1429.

#### General procedure D for the synthesis of compounds **2b-g**.

In a round-bottomed flask, the corresponding compound **4b-g** and dry dichloromethane were cooled to 0°C. Thereafter, a solution of  $\text{BBr}_3$  (C=1.0 M) in dichloromethane was slowly added at 0°C. The reaction mixture was stirred at 20°C for 2h. The suspension was poured into  $\text{EtOAc}$ /Saturated solution of  $\text{NaHCO}_3$ . The organic layer was washed with water and brine, dried over  $\text{MgSO}_4$  and evaporated to dryness to afford compound **2b-g** which was used in the next step without further purification.

*(Z)*-1-(3-Benzyl-5-hydroxybenzothiazol-2(3H)-ylidene)propan-2-one (**2b**). According to the general procedure D, from **4b** (1.0 g, 3.21 mmol) and dry dichloromethane (13 mL). A solution of  $\text{BBr}_3$  (6.42 mL C=1.0 M, 6.42 mmol) in dichloromethane was slowly added at 0 °C. Compound **2b** was obtained (595 mg, 62%) as a brown solid.  $^1\text{H}$  NMR (300 MHz, DMSO)  $\delta$  9.69 (s, 1H), 7.51 (d,  $J = 8.4$  Hz, 1H), 7.45-7.24 (m, 3H), 7.24-7.04 (m, 2H), 6.70 (d,  $J = 2.1$  Hz, 1H), 6.64 (dd,  $J = 8.4, 2.1$  Hz, 1H), 6.14 (s, 1H), 5.34 (s, 2H), 2.03 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  189.52, 160.42, 160.37, 157.14, 156.99, 140.75, 135.34, 128.87, 127.54, 126.45, 122.90, 115.25, 110.95, 98.23, 90.52, 48.08, 28.78, 28.74; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{17}\text{H}_{16}\text{NO}_2\text{S}$ : 298.0902  $[\text{M}+\text{H}]^+$ , found 298.0892.

*(Z)*-1-(5-Hydroxy-3-phenethylbenzo[d]thiazol-2(3H)-ylidene)propan-2-one (**2c**). According to the general procedure D, from **4c** (1.0 g, 3.07 mmol) and dry dichloromethane (13 mL). A solution of  $\text{BBr}_3$  (5.6 mL C=1.0 M, 6.15 mmol) in dichloromethane was slowly added at 0 °C. Compound **2c** was obtained (574 mg, 60%) as a brown solid. m.p. 214-215°C;  $^1\text{H}$  NMR (300 MHz, DMSO)  $\delta$  9.70 (s, 1H), 7.46 (d,  $J = 8.4$  Hz, 1H), 7.39 – 7.15 (m, 5H), 6.74 (d,  $J = 2.1$  Hz, 1H), 6.62 (dd,  $J = 8.4, 2.1$

Hz, 1H), 6.06 (s, 1H), 4.21 (t,  $J = 7.8$  Hz, 2H), 2.95 (t,  $J = 7.8$  Hz, 2H), 2.50 (p,  $J = 1.8$  Hz, 2H), 2.08 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz, DMSO)  $\delta$  189.38, 159.58, 157.09, 140.34, 137.77, 129.02, 128.47, 126.65, 122.70, 115.24, 110.70, 97.88, 90.20, 46.46, 31.85, 28.76; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{18}\text{H}_{18}\text{NO}_2\text{S}$ : 312.1053  $[\text{M}+\text{H}]^+$ , found 312.1068.

*(Z)*-1-(5-Hydroxy-3-(3-methylbenzyl)benzothiazol-2(3H)-ylidene)propan-2-one (**2d**). According to the general procedure D, from **4d** (400 mg, 1.23 mmol), dry dichloromethane (7 mL),  $\text{BBr}_3$  (2.46 mL, 2.46 mmol, C=1.0 M) to obtain **2d** (120 mg, 31%) as a brown solid. m.p. 181-182°C;  $^1\text{H}$  NMR (300 MHz, DMSO)  $\delta$  9.67 (s, 1H), 7.50 (d,  $J = 8.3$  Hz, 1H), 7.23 (t,  $J = 7.6$  Hz, 1H), 7.09 (d,  $J = 7.6$  Hz, 1H), 7.01 (s, 1H), 6.92 (d,  $J = 7.7$  Hz, 1H), 6.71-6.59 (m, 2H), 6.12 (s, 1H), 5.28 (s, 2H), 2.26 (s, 3H), 2.03 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz, DMSO)  $\delta$  189.49, 160.41, 157.07, 140.75, 138.00, 135.24, 128.75, 128.19, 126.87, 123.37, 122.83, 115.23, 110.91, 98.18, 90.45, 48.13, 28.74, 21.07; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{18}\text{H}_{18}\text{NO}_2\text{S}$ : 312.1058  $[\text{M}+\text{H}]^+$ , found 312.1061.

*(Z)*-1-(5-Hydroxy-3-(3-chlorobenzyl)benzothiazol-2(3H)-ylidene)propan-2-one (**2e**). According to the general procedure D, from **4e** (400 mg, 1.16 mmol), dry dichloromethane (7 mL),  $\text{BBr}_3$  (2.31 mL, 2.31 mmol, C=1.0 M) to obtain **2e** (250 mg, 65%) as a brown solid. m.p. 249-250°C;  $^1\text{H}$  NMR (300 MHz, DMSO)  $\delta$  9.70 (s, 1H), 7.52 (d,  $J = 8.4$  Hz, 1H), 7.45-7.31 (m, 2H), 7.26 (dt,  $J = 2.4, 1.1$  Hz, 1H), 7.11-7.01 (m, 1H), 6.71 (d,  $J = 2.1$  Hz, 1H), 6.66 (dd,  $J = 8.4, 2.1$  Hz, 1H), 6.15 (s, 1H), 5.37 (s, 2H), 2.04 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz, DMSO)  $\delta$  189.63, 160.30, 157.17, 140.54, 137.95, 133.36, 130.80, 127.54, 126.37, 124.93, 122.95, 115.19, 111.05, 98.10, 90.54, 47.43, 28.73; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{17}\text{H}_{15}\text{NO}_2\text{S}$ : 332.0512  $[\text{M}+\text{H}]^+$ , found 332.0521.

*(Z)*-1-(5-Hydroxy-3-(4-chlorobenzyl)benzothiazol-2(3H)-ylidene)propan-2-one (**2f**). According to the general procedure D, from **4f** (400 mg, 1.16 mmol), dry dichloromethane (7 mL),  $\text{BBr}_3$  (2.31 mL, 2.31 mmol, C=1.0 M) to obtain **2f** (360 mg, 92%) as a brown solid. m.p. 222-223°C;  $^1\text{H}$  NMR (300 MHz, DMSO)  $\delta$  9.74 (s, 1H), 7.52 (d,  $J = 8.4$  Hz, 1H), 7.48-7.36 (m, 2H), 7.26-7.11 (m, 2H), 6.72 (d,  $J = 2.1$  Hz, 1H), 6.66 (dd,  $J = 8.4, 2.1$  Hz, 1H), 6.14 (s, 1H), 5.35 (s, 2H), 2.03 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz, DMSO)  $\delta$  189.36, 160.46, 157.23, 140.60, 134.37, 132.13, 128.85, 128.40, 122.98, 115.28, 111.14, 98.22, 90.60, 47.47, 28.64; HRMS (ESI):  $m/z$  calcd. for  $\text{C}_{17}\text{H}_{15}\text{NO}_2\text{S}$ : 332.0512  $[\text{M}+\text{H}]^+$ , found 332.0522.

*(Z)*-1-(5-Hydroxy-3-(naphthalen-2-ylmethyl)benzothiazol-2(3H)-ylidene)propan-2-one (**2g**). According to the general procedure D, from **4g** (650 mg, 1.80 mmol), dry dichloromethane (9 mL),  $\text{BBr}_3$  (3.76 mL, 3.76 mmol, C=1.0 M) to obtain **2g** (557 mg, 85%) as a brown solid. m.p. 279-280°C;  $^1\text{H}$  NMR (300 MHz, DMSO)  $\delta$  7.99-7.76 (m, 3H), 7.62 (s, 1H), 7.57-7.40 (m, 3H), 7.32 (dd,  $J = 8.5, 1.8$  Hz, 1H), 6.70 (d,  $J = 2.1$  Hz, 1H), 6.61 (dd,  $J = 8.5, 2.1$  Hz, 1H), 6.16 (s, 1H), 5.45 (s, 2H), 2.01 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz, DMSO)  $\delta$  189.38, 160.52, 158.66, 140.86, 133.00, 132.79, 132.30, 128.61, 127.63, 126.53, 126.12, 124.77, 124.63, 122.73,

114.01, 111.57, 98.43, 90.41, 48.40, 28.77; HRMS (ESI):  $m/z$  calcd. for  $C_{21}H_{18}NO_2S$ : 348.1058 [M+H]<sup>+</sup>, found 348.1064.

*(Z)*-1-(5-Hydroxy-3-(3-methoxybenzyl)benzothiazol-2(3H)-ylidene)propan-2-one (**2h**). To a solution of ester **6** (500 mg, 1.35 mmol) in 30 mL MeOH was added NaOH (54 mg, 1.35 mmol). The reaction was stirred and heated at 65°C for 15 min, then cooled to ambient temperature. A solution of 1 M HCl was added (pH=7) and MeOH was removed under vacuum. The residue was dissolved in 50 mL of water. The precipitate was filtered, washed with water and Et<sub>2</sub>O. The solid was dried to give **2h** as an off-white solid (300 mg, 68%). m.p. 230-231°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 9.69 (s, 1H), 7.51 (d,  $J$  = 8.4 Hz, 1H), 7.27 (d,  $J$  = 7.9 Hz, 1H), 6.86 (ddd,  $J$  = 8.4, 2.6, 0.9 Hz, 1H), 6.81-6.74 (m, 1H), 6.74-6.55 (m, 3H), 6.13 (s, 1H), 5.30 (s, 2H), 3.72 (s, 3H), 2.03 (s, 3H); <sup>13</sup>C NMR (75 MHz, DMSO) δ 189.51, 160.38, 159.50, 157.09, 140.71, 136.86, 130.03, 122.83, 118.27, 115.20, 112.77, 112.35, 110.94, 98.19, 90.47, 55.04, 47.97, 28.74; HRMS (ESI):  $m/z$  calcd. for  $C_{18}H_{18}NO_3S$ : 328.1007 [M+H]<sup>+</sup>, found 328.1008.

#### General procedure E for the synthesis of compounds **3b-g**

To a solution 5-methoxy-2-methylbenzothiazole in 10 mL dry acetonitrile was added the corresponding alkyl halide derivative. The reaction mixture was stirred at 85°C for 18h in a sealed tube. After cooling at 20°C and poured into diethyl ether (30 mL), the precipitate was filtered and washed with diethyl ether (3x10 mL) to yield to the corresponding thiazolium salts **3b-g**.

*3-Benzyl-5-methoxy-2-methylbenzothiazol-3-ium bromide (3b)*. According to the general procedure E, from 5-methoxy-2-methylbenzothiazole (1.79 g, 10 mmol) and benzyl bromide (3.56 mL, 30 mmol). Compound **3b** was obtained as a pale green solid (2.25 mg, 64%) which was not further purified. <sup>1</sup>H NMR (300 MHz, DMSO) δ 8.35 (d,  $J$  = 9.1 Hz, 1H), 7.74 (d,  $J$  = 2.3 Hz, 1H), 7.52-7.24 (m, 6H), 6.07 (s, 2H), 3.86 (s, 3H), 3.19 (s, 3H); <sup>13</sup>C NMR (75 MHz, DMSO) δ 178.05, 160.68, 142.66, 132.97, 129.17, 128.56, 127.19, 125.59, 121.00, 117.73, 100.41, 56.44, 51.73, 17.42; HRMS (ESI):  $m/z$  calcd. for  $C_{16}H_{16}NOS$ : 270.0953 [M-Br]<sup>+</sup>, found 270.0945.

*5-Methoxy-2-methyl-3-phenethylbenzothiazol-3-ium bromide (3c)*. According to the general procedure E, from 5-methoxy-2-methylbenzothiazole (1.0 g, 5.6 mmol), and phenethyl bromide (2.06 g, 11.2 mmol). Compound **3c** was obtained as a greenish white solid (0.492 g, 24%). m.p. 229-230°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 8.32 (d,  $J$  = 9.1 Hz, 1H), 7.69 (d,  $J$  = 2.3 Hz, 1H), 7.38 (dd,  $J$  = 9.1, 2.3 Hz, 1H), 7.24 (dd,  $J$  = 5.2, 1.9 Hz, 3H), 7.15 (dd,  $J$  = 7.0, 2.6 Hz, 2H), 5.03 (t,  $J$  = 6.9 Hz, 2H), 3.93 (s, 3H), 3.22 (t,  $J$  = 6.8 Hz, 2H), 2.88 (s, 3H); <sup>13</sup>C NMR (75 MHz, DMSO) δ 177.05, 160.56, 142.30, 136.80, 129.24, 128.62, 127.17, 125.25, 120.54, 118.01, 99.99, 56.42, 50.34, 33.34, 16.66; HRMS (ESI):  $m/z$  calcd. for  $C_{17}H_{18}NOS$ : 284.1109 [M-Br]<sup>+</sup>, found 284.1109.

*5-Methoxy-3-(3-methylbenzyl)-2-methylbenzothiazol-3-ium bromide (3d)*. According to the general procedure E, from 5-methoxy-2-methylbenzothiazole (1.0 g, 5.6 mmol), and 3-

methylbenzyl bromide (1.51 mL, 11.2 mmol) Compound **3d** was obtained as a white solid (1.87 g, 92%). m.p. 239-240°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 8.40 (d,  $J$  = 9.1 Hz, 1H), 7.76 (d,  $J$  = 2.3 Hz, 1H), 7.41 (dd,  $J$  = 9.0, 2.3 Hz, 1H), 7.28 (t,  $J$  = 7.6 Hz, 1H), 7.22-7.06 (m, 3H), 6.08 (s, 2H), 3.87 (s, 3H), 3.23 (s, 3H), 2.27 (s, 3H); <sup>13</sup>C NMR (75 MHz, DMSO) δ 177.92, 160.60, 142.62, 138.49, 132.81, 129.16, 128.99, 127.56, 125.54, 124.18, 120.93, 117.64, 100.40, 56.41, 51.74, 20.90, 17.46; HRMS (ESI):  $m/z$  calcd. for  $C_{17}H_{18}NOS$ : 284.1109 [M-Br]<sup>+</sup>, found 284.1107.

*5-Methoxy-3-(3-chlorobenzyl)-2-methylbenzothiazol-3-ium bromide (3e)*. According to the general procedure E, from 5-methoxy-2-methylbenzothiazole (0.909 g, 5.07 mmol), and 3-chlorobenzyl bromide (1.45 mL, 10.14 mmol). Compound **3e** was obtained as a white solid (1.25 g, 64%). m.p. 250-251°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 8.39 (d,  $J$  = 9.1 Hz, 1H), 7.73 (d,  $J$  = 2.3 Hz, 1H), 7.53 (dt,  $J$  = 1.8, 1.1 Hz, 1H), 7.48-7.37 (m, 3H), 7.25 (dt,  $J$  = 6.4, 2.0 Hz, 1H), 6.12 (s, 2H), 3.87 (s, 3H), 3.21 (s, 3H); <sup>13</sup>C NMR (75 MHz, DMSO) δ 178.53, 160.69, 142.55, 135.28, 133.66, 130.98, 128.54, 127.20, 125.68, 125.58, 121.01, 117.65, 100.27, 56.42, 51.05, 17.44; HRMS (ESI):  $m/z$  calcd. for  $C_{16}H_{15}NOSCl$ : 304.0563 [M-Br]<sup>+</sup>, found 304.0571.

*5-Methoxy-3-(4-chlorobenzyl)-2-methylbenzothiazol-3-ium bromide (3f)*. According to the general procedure E, from 5-methoxy-2-methylbenzothiazole (1.0 g, 5.6 mmol), and 4-chlorobenzyl bromide (2.29 g, 11.2 mmol). Compound **3f** was obtained as a white solid (1.88 g, 88%). m.p. 244-245°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 8.41 (d,  $J$  = 9.0 Hz, 1H), 7.78 (d,  $J$  = 2.4 Hz, 1H), 7.56-7.28 (m, 5H), 6.16 (s, 2H), 3.88 (s, 3H), 3.23 (s, 3H); <sup>13</sup>C NMR (75 MHz, DMSO) δ 178.19, 160.68, 142.52, 133.14, 131.92, 129.20, 129.01, 125.59, 120.97, 117.71, 100.29, 56.48, 51.02, 17.46; HRMS (ESI):  $m/z$  calcd. for  $C_{16}H_{15}NOSCl$ : 304.0563 [M-Br]<sup>+</sup>, found 304.0554.

*5-Methoxy-2-methyl-3-(naphthalen-2-ylmethyl)benzothiazol-3-ium bromide (3g)*. According to the general procedure E, from 5-methoxy-2-methylbenzothiazole (1.0 g, 5.6 mmol), and 2-(bromomethyl)naphthalene (2.47 g, 11.2 mmol). Compound **3g** was obtained as an off-white solid (1.8 g, 81%). m.p. 203-204°C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 8.41 (d,  $J$  = 9.1 Hz, 1H), 8.05-7.76 (m, 5H), 7.52 (ddd,  $J$  = 8.7, 4.4, 2.1 Hz, 3H), 7.42 (dd,  $J$  = 9.1, 2.3 Hz, 1H), 6.28 (s, 2H), 3.85 (s, 3H), 3.27 (s, 3H); <sup>13</sup>C NMR (75 MHz, DMSO) δ 178.24, 160.68, 142.71, 132.73, 132.53, 130.43, 128.88, 127.85, 127.63, 126.71, 126.67, 125.87, 125.56, 124.82, 121.03, 117.66, 100.41, 56.39, 51.92, 17.43; HRMS (ESI):  $m/z$  calcd. for  $C_{20}H_{18}NOS$ : 320.1109 [M-Br]<sup>+</sup>, found 320.1116.

#### General procedure F for the synthesis of compounds **4b-g**

In a tube, the corresponding salt **3b-g** was suspended in dry acetonitrile (10 mL). Then, Ac<sub>2</sub>O and Et<sub>3</sub>N were successively added. The tube was sealed and the resulting solution was heated at 85°C for 18 hours. Thereafter, the solution was cooled to 20°C and the solvent was removed under vacuum. The residue was dissolved in ethyl acetate (100 mL), washed with water and brine. The organic layer was dried on MgSO<sub>4</sub> and evaporated under vacuum. The residue obtained was purified

by flash chromatography on silica gel to afford the expected compound **4b-g**.

*(Z)*-1-(3-Benzyl-5-methoxybenzothiazol-2(3H)-ylidene)propan-2-one (**4b**). According to the general procedure F, from **3b** (1.75 g, 5 mmol), Ac<sub>2</sub>O (1.1 mL, 11.5 mmol) and Et<sub>3</sub>N (1.7 mL, 12 mmol). Purification by flash chromatography on silica gel (EtOAc/Petroleum ether gradient from 1:9 to 3:7) afforded **4b** (1.22 g, 78%) as a brown solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.48 (d, *J* = 8.5 Hz, 1H), 7.42-7.28 (m, 3H), 7.22-7.11 (m, 2H), 6.76 (dd, *J* = 8.5, 2.2 Hz, 1H), 6.59 (d, *J* = 2.3 Hz, 1H), 5.88 (s, 1H), 5.17 (s, 2H), 3.78 (s, 3H), 2.17 (s, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 191.56, 161.74, 159.53, 141.10, 134.12, 129.24, 128.12, 126.27, 122.89, 118.68, 109.04, 96.93, 91.13, 55.84, 49.37, 29.14; HRMS (ESI): *m/z* calcd. for C<sub>18</sub>H<sub>18</sub>NO<sub>2</sub>S: 312.1058 [M+H]<sup>+</sup>, found 312.1057.

*(Z)*-1-(5-Methoxy-3-phenethylbenzothiazol-2(3H)-ylidene)propan-2-one (**4c**). According to the general procedure F, from **3c** (0.436 g, 1.20 mmol), Ac<sub>2</sub>O (0.26 mL, 2.75 mmol) and Et<sub>3</sub>N (0.49 mL, 3.59 mmol). Purification by flash chromatography on silica gel (Et<sub>2</sub>O/ EtOAc/Petroleum ether 4:3:3) afforded **4c** (0.304 g, 78%) as a light brown solid. m.p. 164-165 °C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.39 (d, *J* = 8.5 Hz, 1H), 7.34-7.15 (m, 5H), 6.70 (dd, *J* = 8.5, 2.2 Hz, 1H), 6.43 (d, *J* = 2.3 Hz, 1H), 5.84 (s, 1H), 4.13 (dd, *J* = 8.4, 6.8 Hz, 2H), 3.76 (s, 3H), 3.03 (t, *J* = 7.6 Hz, 2H), 2.22 (s, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 191.11, 161.01, 159.29, 140.61, 137.55, 128.98, 128.82, 127.17, 122.69, 118.55, 108.99, 96.36, 90.45, 55.77, 47.38, 32.74, 29.12; HRMS (ESI): *m/z* calcd. for C<sub>19</sub>H<sub>20</sub>NO<sub>2</sub>S: 326.1215 [M+H]<sup>+</sup>, found 326.1213.

*(Z)*-1-(5-Methoxy-3-(3-methylbenzyl)benzothiazol-2(3H)-ylidene)propan-2-one (**4d**). According to the general procedure F, from **3d** (1.0 g, 2.74 mmol), Ac<sub>2</sub>O (0.597 mL, 6.31 mmol) and Et<sub>3</sub>N (1.15 mL, 8.23 mmol). Purification by flash chromatography on silica gel (Et<sub>2</sub>O/ EtOAc/Petroleum ether 3:3:4) afforded **4d** (0.783 g, 88%) as a light brown solid. m.p. 147-148 °C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.44 (dd, *J* = 8.5, 1.0 Hz, 1H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.09 (d, *J* = 7.6 Hz, 1H), 6.95 (d, *J* = 6.5 Hz, 2H), 6.74 (ddd, *J* = 8.6, 2.3, 0.7 Hz, 1H), 6.58 (d, *J* = 2.3 Hz, 1H), 5.86 (s, 1H), 5.11 (s, 2H), 3.76 (d, *J* = 0.8 Hz, 3H), 2.30 (s, 3H), 2.15 (s, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 191.45, 161.71, 159.50, 141.13, 139.02, 134.07, 129.06, 128.84, 126.75, 123.28, 122.76, 118.63, 109.02, 96.93, 91.08, 55.78, 49.36, 29.08, 21.52; HRMS (ESI): *m/z* calcd. for C<sub>19</sub>H<sub>20</sub>NO<sub>2</sub>S: 326.1215 [M+H]<sup>+</sup>, found 326.1217.

*(Z)*-1-(5-Methoxy-3-(3-chlorobenzyl)benzothiazol-2(3H)-ylidene)propan-2-one (**4e**). According to the general procedure F, from **3e** (1.0 g, 2.60 mmol), Ac<sub>2</sub>O (0.57 mL, 6 mmol) and Et<sub>3</sub>N (1.09 mL, 7.80 mmol). Purification by flash chromatography on silica gel (Et<sub>2</sub>O/EtOAc/Petroleum ether 3:3:4) afforded **4e** (0.591 g, 66%) as a light brown solid. m.p. 170-171 °C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.45 (d, *J* = 8.5 Hz, 1H), 7.29-7.20 (m, 2H), 7.18-7.12 (m, 1H), 7.01 (ddd, *J* = 4.9, 3.4, 1.7 Hz, 1H), 6.75 (dd, *J* = 8.5, 2.2 Hz, 1H), 6.53 (d, *J* = 2.2 Hz, 1H), 5.81 (s, 1H), 5.11 (s, 2H), 3.77 (s, 3H), 2.15 (s, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 191.58,

161.47, 159.60, 140.84, 136.25, 135.21, 130.56, 128.40, 126.37, 124.35, 122.95, 118.57, 109.08, 96.82, 91.14, 55.86, 48.96, 29.15; HRMS (ESI): *m/z* calcd. for C<sub>18</sub>H<sub>17</sub>NO<sub>2</sub>SCl: 346.0669 [M+H]<sup>+</sup>, found 346.0673.

*(Z)*-1-(5-Methoxy-3-(4-chlorobenzyl)benzothiazol-2(3H)-ylidene)propan-2-one (**4f**). According to the general procedure F, from **3f** (1.0 g, 2.60 mmol), Ac<sub>2</sub>O (0.565 mL, 6 mmol) and Et<sub>3</sub>N (1.09 mL, 7.80 mmol). Purification by flash chromatography on silica gel (Et<sub>2</sub>O/EtOAc/Petroleum ether 3:3:4) afforded **4f** (0.285 g, 32%) as a light brown solid. m.p. 188-189 °C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.47 (d, *J* = 8.5 Hz, 1H), 7.36-7.27 (m, 2H), 7.14-7.06 (m, 2H), 6.76 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.54 (d, *J* = 2.2 Hz, 1H), 5.82 (s, 1H), 5.12 (s, 2H), 3.78 (s, 3H), 2.16 (s, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 191.61, 161.58, 159.63, 140.91, 134.06, 132.64, 129.49, 127.70, 123.02, 118.71, 109.06, 96.90, 91.14, 55.90, 48.77, 29.19; HRMS (ESI): *m/z* calcd. for C<sub>18</sub>H<sub>17</sub>NO<sub>2</sub>SCl: 346.0669 [M+H]<sup>+</sup>, found 346.0670.

*(Z)*-1-(5-Methoxy-3-(naphthalen-2-ylmethyl)benzothiazol-2(3H)-ylidene)propan-2-one (**4g**). According to the general procedure F, from **3g** (1.5 g, 3.75 mmol), Ac<sub>2</sub>O (0.82 mL, 8.62 mmol) and Et<sub>3</sub>N (1.57 mL, 11.24 mmol). Purification by flash chromatography on silica gel (Et<sub>2</sub>O/EtOAc/Petroleum ether 3:3:4) afforded **4g** (0.917 g, 68%) as a light brown solid. m.p. 175-176 °C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.84 (dd, *J* = 9.0, 5.7 Hz, 2H), 7.74 (dd, *J* = 6.1, 3.5 Hz, 1H), 7.53 (s, 1H), 7.51-7.44 (m, 3H), 7.32 (dd, *J* = 8.5, 1.9 Hz, 1H), 6.76 (dd, *J* = 8.5, 2.2 Hz, 1H), 6.62 (d, *J* = 2.2 Hz, 1H), 5.92 (s, 1H), 5.32 (s, 2H), 3.75 (s, 3H), 2.15 (s, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 191.62, 161.77, 159.60, 141.17, 133.44, 133.02, 131.49, 129.28, 127.92, 127.86, 126.75, 126.43, 124.95, 124.03, 122.92, 118.74, 109.11, 97.00, 91.24, 55.83, 49.63, 29.16; HRMS (ESI): *m/z* calcd. for C<sub>22</sub>H<sub>20</sub>NO<sub>2</sub>S: 362.1215 [M+H]<sup>+</sup>, found 362.1208.

*5-Hydroxy-3-(3-methoxybenzyl)-2-methylbenzothiazol-3-ium bromide (5)*. To a solution of 5-hydroxy-2-methylbenzothiazole (1.0 g, 6.05 mmol) in 10 mL dry acetonitrile was added 3-methoxybenzyl bromide (1.70 mL, 12.1 mmol). The reaction mixture was stirred at 85 °C for 18h in a sealed tube. After cooling at 20 °C and pouring into diethyl ether (30 mL), the precipitate was filtered and washed with diethyl ether (3x10 mL) to yield **5** as an off-white solid (1.95 g, 88%). m.p. 218-219 °C; <sup>1</sup>H NMR (300 MHz, DMSO) δ 10.65 (s, 1H), 8.29 (d, *J* = 8.9 Hz, 1H), 7.41 (d, *J* = 2.2 Hz, 1H), 7.36-7.20 (m, 2H), 6.93 (dq, *J* = 10.6, 1.5, 0.9 Hz, 2H), 6.85-6.69 (m, 1H), 5.95 (s, 2H), 3.75 (s, 3H), 3.24 (s, 3H); <sup>13</sup>C NMR (75 MHz, DMSO) δ 177.51, 159.64, 158.99, 142.54, 134.29, 130.38, 125.53, 119.18, 118.61, 118.18, 113.55, 113.21, 102.04, 55.24, 51.68, 17.37; HRMS (ESI): *m/z* calcd. for C<sub>16</sub>H<sub>16</sub>NO<sub>2</sub>S: 286.0902 [M-Br]<sup>+</sup>, found 286.0903.

*(Z)*-3-(3-Methoxybenzyl)-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl acetate (**6**). In a tube, salt **5** (1.0 g, 2.60 mmol) was suspended in dry acetonitrile (15 mL). Then, Ac<sub>2</sub>O (1.19 mL, 12.6 mmol) and Et<sub>3</sub>N (2.85 mL, 16.4 mmol) were successively added. The tube was sealed and the resulting solution was heated at 85 °C for 18 hours. Thereafter, the



solution was cooled to 20°C and the solvent was removed under vacuum. The residue was dissolved in ethyl acetate (100 mL), washed with water and brine. The organic layer was dried on MgSO<sub>4</sub> and evaporated under vacuum. The light brown solid (0.730 g, 72%) was used in the next step without further purification. <sup>1</sup>H NMR (300 MHz, DMSO) δ 7.77 (d, *J* = 9 Hz, 1H), 7.28–7.22 (m, 2H), 6.98 (dd, *J* = 9.0, 3.0 Hz, 1H), 6.86 (m, 1H), 6.78 (m, 1H), 6.68 (dd, *J* = 9.0, 3.0 Hz, 1H), 6.15 (s, 1H), 5.34 (s, 2H), 3.71 (s, 3H), 2.25 (s, 3H), 2.06 (s, 3H). HRMS (ESI): *m/z* calcd. for C<sub>20</sub>H<sub>20</sub>NO<sub>4</sub>S: 370.1113 [M+H]<sup>+</sup>, found 370.1097.

*Bis((Z)-3-ethyl-2-(2-oxopropylidene)-2,3-dihydrobenzothiazol-5-yl) carbonate (7)*. INDY **2a** (367 mg, 1.56 mol) and sodium hydride (60% suspension in mineral oil, 66 mg, 1.64 mmol) were dissolved in dry dimethylformamide (15 mL). After 10 min a bright yellow solution was obtained and *N,N'*-disuccinimidyl carbonate (200 mg, 0.781 mmol) was added. The mixture was stirred at 20°C for 1h to afford a milky grey suspension. The reaction was then carefully hydrolyzed with water and extracted three times with dichloromethane. The organic layers were washed with brine, dried over MgSO<sub>4</sub> and evaporated to dryness. The resulting solid was purified by flash chromatography on silica gel (EtOAc/PE 1:1) to afford **7** (315 mg, 81%) as a beige solid. m.p. 205 °C (decomp.); <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 7.82 (d, *J* = 8.4 Hz, 1H), 7.58 (d, *J* = 1.9 Hz, 2H), 7.22 (dd, *J* = 8.5, 2.0 Hz, 2H), 6.17 (s, 2H), 4.16 (q, *J* = 7.1 Hz, 4H), 2.13 (s, 6H), 1.24 (t, *J* = 7.0 Hz, 6H); <sup>13</sup>C NMR (75 MHz, DMSO) δ 190.28, 159.02, 151.74, 150.02, 140.04, 124.09, 123.10, 115.60, 104.12, 90.67, 28.85, 11.51; IR (neat, ν) 2933, 1766, 1599, 1444, 1224, 1186, 973; HRMS (ESI): *m/z* calcd. for C<sub>25</sub>H<sub>25</sub>N<sub>2</sub>O<sub>5</sub>S<sub>2</sub>: 497.1199 [M+H]<sup>+</sup>, found 497.1203.

**In vitro studies.** Inhibition studies for human kinases (*hDYRK1A/hCLK1*) were performed by the Eurofins company. The AChE and BuChE biological activities, the blood-brain barrier permeability (PAMPA-BBB Assay) and chromatographic hydrophobicity index (CHI) LogD<sub>7.4</sub> of the synthesized compounds were evaluated by the DruID platform at the CERMN research center (UR 4258, UNICAEN).

**AChE and BuChE biological activity.** Inhibitory capacity of compounds on AChE biological activity was evaluated through the use of the spectrometric method of Ellman. Acetyl- or butyrylthiocholine iodide and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) were purchased from Sigma Aldrich. Lyophilized BuChE from equine serum (Sigma Aldrich) was dissolved in 0.2 M phosphate buffer pH 7.4 such as to have enzyme solutions stock with 2.5 units/mL enzyme activity. AChE from human erythrocytes (buffered aqueous solution, ≥500 units/mg protein (BCA), Sigma Aldrich) was diluted in 20 mM HEPES buffer pH 8, 0.1% Triton X-100 such as to have enzyme solution with 0.25 units/mL enzyme activity. In the procedure, 100 μL of 0.3 mM DTNB dissolved in phosphate buffer pH 7.4 were added into the 96 wells plate followed by 50 μL of the tested compound solution and 50 μL of enzyme solution. After 5 min of preincubation, the reaction was then initiated by the injection of 50 μL of 10 mM acetyl- or butyrylthiocholine iodide solution.

The hydrolysis of acetyl- or butyrylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetyl- or butyrylthiocholine, at a wavelength of 412 nm every minute for 10 min using a 96-well microplate plate reader (TECAN Infinite® M200, Lyon, France). Tested compounds were dissolved in analytical grade DMSO. Donepezil and Tacrine were used as reference standards (Donepezil for AChE and Tacrine for BuChE). First screening of BuChE activity and AChE from human erythrocytes activity were carried out at a 10<sup>-5</sup> M, 10<sup>-5</sup> M and at 10<sup>-6</sup> M concentration of compounds respectively under study. For the compounds with significant inhibition (≥ 50%) after 4 min of reaction, IC<sub>50</sub> values were determined graphically from 6 points inhibition curves using the Origin® software.

**DYRK1A Human CMGC Kinase Enzymatic Radiometric Assay [Km ATP].** *hDYRK1A* is incubated with 8 mM MOPS pH 7.0, 0.2 mM EDTA, 50 μM RRRFRPASPLRGPPK, 10 mM MgAcetate and [gamma-33P]-ATP (specific activity and concentration as required). The reaction is initiated by the addition of the Mg/ATP mix. After incubation for 40 min at 20°C, the reaction is stopped by the addition of phosphoric acid to a concentration of 0.5%. An aliquot of the reaction is then spotted onto a filter and washed four times for 4 minutes in 0.425% phosphoric acid and once in methanol prior to drying and scintillation counting. Staurosporine was used as a control inhibitor.

**CLK1 Human CMGC Kinase Enzymatic Radiometric Assay [Km ATP].** *hCLK1* is incubated with 8 mM MOPS pH 7.0, 0.2 mM EDTA, 1 mM sodium orthovanadate, 5 mM sodium 6-glycerophosphate, 200 μM ERMRPRKRQGSVRRRV, 10 mM MgAcetate and [gamma-33P]-ATP (specific activity and concentration as required). The reaction is initiated by the addition of the Mg/ATP mix. After incubation for 40 minutes at room temperature, the reaction is stopped by the addition of phosphoric acid to a concentration of 0.5%. An aliquot of the reaction is then spotted onto a filter and washed four times for 4 minutes in 0.425% phosphoric acid and once in methanol prior to drying and scintillation counting. Staurosporine was used as a control inhibitor.

**PAMPA-BBB assay.**<sup>35,36</sup> The PAMPA-BBB test is performed according to the methodology using the BBB-Pampa Explorer® kit (pION Inc, Woburn, MA, USA). It consists in the measurement of the flow rate of a compound from a compartment to another through an artificial membrane at pH=7.4. Compounds were diluted to 20 mM in DMSO and then at 100 μM in pH 7.4 Prisma HT buffer (pION). Then, 200 μL of this solution were added to each well of the donor plate (*n* = 6). The filter membrane on the acceptor plate was coated with 5 μL of the BBB-1 lipid (pION) formulation and to each well of the acceptor plate, 200 μL of brain sink buffer (BSB, pION) was added. The acceptor filter plate was placed on top of the donor plate to form a “sandwich”. The sandwich was incubated at room temperature

for 4h without stirring. The sandwich is then separated and the UV-vis spectra were measured in the reference, acceptor and donor plates using a microplate reader (Tecan infinite® M200). For each compound,  $-\log P_e$  was calculated using the PAMPA Explorer® software v. 3.7 (pION). The references used are theophylline (250  $\mu\text{M}$ ,  $P_e(10^{-6}\text{cm/s})=1.1\pm 0.1$ , prediction CNS-) and corticosterone (100  $\mu\text{M}$ ,  $P_e(10^{-6}\text{cm/s})=153.3\pm 7.2$ , prediction CNS+). The result obtained for **1b** (100  $\mu\text{M}$ ) is  $P_e(10^{-6}\text{cm/s})=187.2\pm 52.6$  corresponding to a prediction CNS+.

**Chromatographic hydrophobicity index (CHI)  $\text{Log}D_{7.4}$ .** The determination of Chromatographic Hydrophobicity Index (CHI) at neutral pH was realized by retention time measurements using a 1290 Agilent chromatographic system (Agilent Technologies, Santa Clara, California, USA) equipped with a PDA detector 1260 operating at 220, 240, 254, 290 and 350 nm for all compounds using Ultra C18 column (5  $\mu\text{m}$ , 3x50 mm) from Restek (Lisses, France). Before measurements, compounds were dissolved (100  $\mu\text{M}$ ) in methanol. In the used HPLC method, the flow rate was 0.3  $\text{mL}\cdot\text{min}^{-1}$ , the temperature was kept constant at 30°C. The mobile phase A was made of 50 mM ammonium acetate aqueous solution, and the mobile phase B was acetonitrile. A HPLC gradient method was applied, with 0 to 0.2 min: 0% B solvent; 0.2 to 3.5 min: 0 to 100% B solvent; 3.5 to 4.0 min: 100% B solvent; post-run: 2 min. Standard compounds were theophylline, 5-Phenyltetrazole, benzimidazole, acetophenone, indole, propiophenone, butyrophenone, valerophenone, with  $R^2>0.999$ . The Chromatographic Hydrophobicity data expressed as CHI  $\text{Log}D$  was calculated for **1b** (CHI  $\text{Log}D=3.00$ , CHI 7.4=82.4) and **1f** (CHI  $\text{Log}D=4.36$ , CHI 7.4=108.8).

**Solubility of **1b**.** The analysis was performed using a LC system (Thermo U3000) under UV detection using the parameters optimized for each compound. HPLC analysis was performed using Accucore RP-MS C18 (150x2.1 mm, 2.6  $\mu\text{m}$ ); the gradient (ACN in ammonium acetate buffer 20 mM) and the mobile phase (flow rate 400  $\mu\text{L}\cdot\text{min}^{-1}$ ) used are determined in order to detect the compound of interest with satisfying retention time and peak shape (run time 35 min, column temperature 30°C). Acquisition and analysis of data were performed with Chromeleon software. 10  $\mu\text{L}$  of a 10 mM solution in DMSO of the compound are diluted either in 490  $\mu\text{L}$  of PBS pH 7.4 or in MeOH in a pre-labelled micro centrifuge tube (in triplicate). The tubes are shaken 24h at room temperature, then centrifuged at 3400 rpm for 5 min. Then, the mixtures are filtered over 0.45  $\mu\text{m}$  filters and 10  $\mu\text{L}$  of samples were subjected to HPLC analysis. The solubility is determined by measuring the ratio of mass signal area PBS/organic solvent.

**HPLC decarbamylation study of **1b**.** The HPLC study was performed on a Thermo Scientific™ - UltiMate™ 3000 LC Systems equipped with a reversed phase column from Waters (ACQUITY UPLC® BEH C18, 100\*2.1 mm, 1.7  $\mu\text{m}$ ). The mobile

phase was composed of a mixture of water (0.1% formic acid) and acetonitrile using a gradient mode from 5%  $\text{CH}_3\text{CN}$  to 100%  $\text{CH}_3\text{CN}$  over 9.5 minutes, the injection volumes was 1  $\mu\text{L}$  and the flow rate 0.4  $\text{mL}/\text{min}$ . The *eq*BuChE enzyme solution was prepared by diluting commercial solid protein (1.21 mg, 1007 units) with PBS buffer (1.0 mL, pH 7.4). Next, 1.5 mg of compound **1b** were dissolved in 10  $\mu\text{L}$  DMSO and 990  $\mu\text{L}$  PBS buffer (pH 7.4) to give a stock solution of 3.92 mM concentration. To the *eq*BuChE enzyme solution was added 50  $\mu\text{L}$  of the **1b** stock solution (final concentration 0.187 mM) and the mixture was incubated at 37 °C with continuous shaking. The progression of the reaction was checked by HPLC every 24h. For this, 200  $\mu\text{L}$  aliquots of the reaction solution were taken and 200  $\mu\text{L}$  of  $\text{CH}_3\text{CN}$  were added to precipitate the enzyme. A clear solution was obtained by filtration by centrifugation using Amicon Ultra 0.5 filters. A volume of 1  $\mu\text{L}$  was injected for analysis.

**Molecular docking.** The initial model of compounds **1c**, **1f** and **2c** was built starting from INDY X-ray structure from the 3ANQ complex using BIOVIA Discovery Studio v19.1 and its protonation state at pH 7.4 was predicted using standard tools of the ChemAxon Package.<sup>40</sup> The majority non-protonated microspecies for the three compounds was used for docking studies.

#### *Equine butyrylcholinesterase.*

To do the docking studies on the equine butyrylcholinesterase first a homology model was built. The sequence of the equine butyrylcholinesterase was retrieved from the UniProt Knowledgebase (UniProtKB)<sup>41-50</sup> (ID: P81908\_HORSE). The homology model was built using @tome server<sup>47</sup> and as a template we selected the X-ray structure of human butyrylcholinesterase (Sequence identity = 90%) in complex with (*S*)-*N*-(1-((2-cycloheptylethyl)amino)-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl)-*N,N*-dimethylbutan-1-aminium (PDB ID 6QAB, a structure refined to 2.49Å with an R free factor of 23.6%<sup>51</sup>). The automatic alignment generated by @tome server was used as the basis for the homology modeling with the Modeller software.<sup>48</sup> The *eq*BuChE amino acid protonation state was checked before the docking study using the PropKA software<sup>52</sup> and the proposed protonation for Glu197 was applied. The docking of the compound into the BuChE binding site was carried out with the GOLD program (2020.2.0) using the default parameters.<sup>53,54</sup> This program applies a genetic algorithm to explore conformational spaces and ligand binding modes. To evaluate the proposed ligand positions, the ChemPLP fitness function was applied. The binding site in the BuChE model was defined as a 7 Å sphere from the co-crystallized ligand. The Ser198 and Glu197 side chains were kept flexible (library option) and a hydrogen bond constraint between the Ser198 side chain and the carbonyl oxygen of the ligand was applied during the docking.

#### *Human DYRK1A and CLK1.*

The crystallographic coordinates of *human* DYRK1 used in this study were obtained from the X-ray structure of the DYRK1 co-crystallized with INDY inhibitor (PDB ID 3ANQ,<sup>25</sup> a structure refined to 2.60 Å with an R-free factor of 27.0%) and those of *human* CLK1 from the X-ray structure of the CLK1 bound with benzothiazole TG003 inhibitor, an INDY derivative (PDB ID 6YTE,<sup>38</sup> a structure refined to 2.30 Å with an R-free factor of 25.8%).

The docking of the compound **2c** into the *h*CLK1 and *h*DYRK1 was carried out with the GOLD program (2020.2.0) using the default parameters.<sup>53,54</sup> The binding site in both kinases was defined as a 6Å sphere from the co-crystallized ligand and the ChemPLP fitness function was applied to evaluate the docking results.

### Conflicts of interest

The authors declare no competing financial interest.

### Acknowledgements

This work was partially supported by University of Rouen Normandy, INSA Rouen Normandy, the Centre National de la Recherche Scientifique (CNRS), European Regional Development Fund (ERDF), Labex SynOrg (ANR-11-LABX-0029), Carnot Institute I2C, the graduate school for research XL-Chem (ANR-18-EURE-0020XL CHEM), and by Région Normandie.

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View Article Online  
DOI: 10.1039/D3MD00708A

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