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Switching Reversibility to Irreversibility in Glycogen Synthase Kinase 3 Inhibitors: Clues for Specific Design of New Compounds

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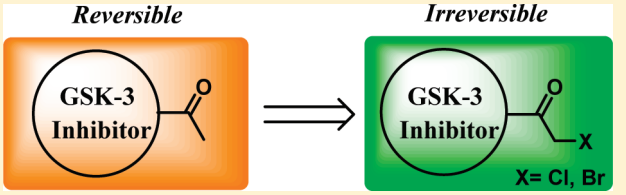
Supporting Information

ABSTRACT: Development of kinase-targeted therapies for central nervous system (CNS) diseases is a great challenge. Glycogen synthase kinase 3 (GSK-3) offers a great potential for severe CNS unmet diseases, being one of the inhibitors on clinical trials for different tauopathies. Following our hypothesis based on the enhanced reactivity of residue Cys199 in the binding site of GSK-3, we examine here the suitability of phenylhalomethylketones as irreversible inhibitors. Our data confirm that the halomethylketone unit is essential for the inhibitory activity. Moreover, addition of the halomethylketone moiety to reversible inhibitors turned them into irreversible inhibitors with IC_{50} values in the nanomolar range. Overall, the results point out that these compounds might be useful pharmacological tools to explore physiological and pathological processes related to signaling pathways regulated by GSK-3 opening new avenues for the discovery of novel GSK-3 inhibitors.



INTRODUCTION

Protein kinases are important targets for cancer, inflammatory diseases, diabetes, and neurodegenerative disorders, because aberrant protein kinase signaling is implicated in many of these human diseases.[1] To date, ten protein kinase (PK) inhibitors are in clinical use for different cancer treatments.[2] While some of these agents are active in a subset of patients, most of them develop resistance within the course of one year. To overcome such a problem, irreversible PK inhibitors are gaining importance[3] as a new class of therapeutic agents. This knowledge is now being applied to other therapeutic areas such as inflammatory and neurodegenerative pathologies, where PK inhibitors have not reached clinical use yet.

Although several candidate therapeutics for PKs in the central nervous system (CNS) are now in different preclinical and clinical stages,[4] the development of kinase-targeted therapies for CNS diseases remains a challenge. Specifically inhibition of kinases that phosporylate tau protein could be beneficial for neurodegenerative diseases.[5] Among them, glycogen synthase kinase 3 (GSK-3) and cyclin-dependent kinase 5 (CDK-5) play a major hyperphosphorylating role *in vivo*.[6] GSK-3 inhibitors offer a valuable approach for a future therapy against Alzheimer's disease (AD).[7−9] Thus, abnormally high GSK-3 activity found in AD patient brains accounts not only for tau hyperphosphorylation, but also for memory impairment, increased β-amyloid production and local plaque-associated microglial-mediated inflammatory responses, which are characteristic hallmarks of the disease.[10] Recently, the reversion of these pathological features *in vivo* has been shown after oral treatment with a GSK-3 inhibitor in a double transgenic animal model of AD.[11]

Many drug discovery programs are focused on the development of selective GSK-3 inhibitors. The discovery of non ATP-competitive inhibitors with high GSK-3 specificity is particularly challenging in order to minimize unfavorable off-target effects. In this context, allosteric GSK-3 modulation, as suggested for the basic alkaloid manzamine,[12,13] or GSK-3 substrate competition,[14] as noted for a small peptide in a study for diabetes, have been valuable strategies to modulate the activity of GSK-3. Other approaches to target GSK-3 avoiding ATP competition have also been envisaged. Specifically, on the basis of previous studies on GSK-3 inhibitors,[15,16] we have recently hypothesized a new way to selectively target this important kinase based on the existence of a cysteine (Cys199) located in the ATP binding site.[17] Since Cys199 in GSK-3 is replaced by different amino acids in other structurally related kinases, such as CDK-1, CDK-2, or CDK-5, this approach would offer the possibility to design specific drugs via covalent modification of this key residue. Indirect support for this strategy comes from the inhibitory activity determined for thienylhalomethylketones and thiadiazolidindiones (TDZDs),[18] which are the first reported non ATP-competitive GSK-3 inhibitors.[19] Among TDZDs, tideglusib (formerly known as NP-12) is now

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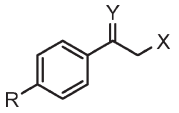
Table 1. GSK-3 Inhibitory Activity of HMKs and Related Compounds



no	X	R	Y	IC_{50} (μM)
1[a]	Cl	H	O	50
2[a]	Br	H	O	5.0
3[a]	Cl	Cl	O	2.5
4[a]	Br	Cl	O	1.0
5[a]	Br	Br	O	0.5
6[a]	Br	Me	O	2.5
7[a]	Br	Ph	O	2.5
8[a]	Br	OMe	O	1.0
9[a]	Br	NO_2	O	2.0
10	I	Br	O	3.0
11	phthalimide	Br	O	100
12	NH_2	Br	O	100
13	SEt	Br	O	>10
14	SPh	Br	O	>100
15	SBn	Br	O	>10
16	OCOMe	Br	O	>100
17	OH	Br	O	>100
18	Br	Br	NOH	>100
19	Br	Br	NOMe	50
20	Cl	Br	SO	>100

[a] See ref 21

in phase II clinical trials both for AD and for an orphan tauopathy called progressive supranuclear palsy (PSP).

Simultaneously with the writing of this manuscript, some data underlying the importance of cysteine identification on the kinase nucleotide binding site for the design of covalent specific protein kinase inhibitors have been reported.[20]

Our aim here is to examine the suitability of targeting Cys199, at the entrance of the ATP binding site of GSK-3, as a new drug discovery strategy to develop compounds useful as selective pharmacological tools against this important protein kinase. To this end, we first report the synthesis and biological characterization of phenylhalomethylketones chosen as bioisosters of the irreversible inhibitors previously described in our studies.[17,21] Moreover, based on X-ray crystallographic and molecular modeling data of GSK-3 complexes, the suitability of our strategy is reinforced by switching compounds with reversible inhibitory properties into irreversible inhibitors. This strategy led us to design, synthesize, and evaluate chemically diverse GSK-3 inhibitors with different kinetics regarding this enzyme. Overall, the results support the implications of Cys199 in the irreversible inhibition of GSK-3 and discover new clues for the synthesis of novel inhibitors for the treatment of human neurodegenerative diseases.

RESULTS AND DISCUSSION

Lead Modification and Structure−Activity Relationship. Screening of our compound library led to the discovery of thienylhalomethylketones as GSK-3 inhibitors.[21] This behavior was reinforced by the inhibitory activity found for a small set of commercially available 4-substituted chloro and bromoacetylphenyl derivatives (**1**−**9**), as expected from the known bioisosterism between thiophene and benzene rings. Although these compounds showed an interesting range of activities, no clear relationship could be identified between the chemical features of the *para*-substitution and the biological activity (Table 1).

To gain insight into the structure−activity relationships of phenylhalomethylketones as GSK-3 inhibitors, a chemically more diverse set of molecules was evaluated using the 4-bromo-substituted derivative **5** as lead compound due to its high inhibitory activity (IC_{50} = 0.5 μM). The novel derivatives were designed to explore the influence played by the nature of the halide atom and/or the carbonyl group, which were replaced by different heteroatoms or moieties such as iodine (**10**), oxygen (**16**, **17**), nitrogen (**11**, **12**), sulfur (**13**−**15**), sulfur monoxide (**20**), oxime (**18**), and alkyloxime (**19**) (Scheme 1).

The GSK-3 inhibitory activity was measured following a previously described method that uses [γ-^{32}P]ATP and the small peptide GS-1 (based on residues of glycogen synthase phosphorylated by GSK-3) as substrate.[22] Among the different chemical modifications examined here, only replacement of the bromo acetyl moiety in compound **5** by the iodoacetyl one in **10** retained the inhibitory activity (IC_{50} = 3.0 μM). All the other compounds did not show significant inhibitory activity (Table 1). Therefore, these findings point out that the halomethylketone (HMK) moiety is a chemical feature necessary for the inhibition of GSK-3.

To further examine the influence of the substituent in the benzene ring, different commercial acetophenones were converted to their bromomethylketo derivatives (**21**, **23**, **25**, and **27**) by bromine treatment in chloroform at low temperature (Scheme 2). In some cases, the dibromo methyl keto compound (**22**, **24**, **26**, and **28**) was also isolated. When *p*-aminoacetophenone was used as starting material, bromination not only in the methylketone group but also in the benzene ring was obtained (**29**). Furthermore, the benzene ring was replaced by the π-deficient 2-pyridine heterocycle, and bromination of 2-acetylpyridine yielded the dibromo derivative **30**. In all cases, an IC_{50} value in the micromolar range was found (Table 2), thus reinforcing the assumption that the bromoacetyl moiety is the key structural feature responsible for the inhibitory activity.

Chemical Reactivity of Phenylhalomethylketones versus Thiol Group. It can be hypothesized that the GSK-3 inhibitory activity of haloacetylphenyl derivatives stems from the reactivity of the HMK moiety with the thiol unit of Cys199, which is located in the ATP binding site. It is therefore necessary to characterize the potential reactivity of haloacetylphenyl compounds in front of thiol groups. To this end, we have analyzed by HPLC−MS the reaction of compound **3** or **4** with 2-mercaptoethanol at room temperature (Table 3). The reaction afforded 11% and 20% of the S-alkylated product for **3** and **4**, respectively, in a 1:1 mix ratio after 1 h. When the mixture was stirred for 18 h, the yield of the S-alkylation was 74% and 50%, respectively. When the concentration of mercaptoethanol was increased 10-fold, 78% and 100% of the S-alkylated product was formed after 18 h. On the other hand, upon addition of a tertiary amine, such as triethylamine, at equimolar concentrations of compound **3** or **4** and mercaptoethanol, the S-alkylated product was formed in 65% (**3**) and 66% (**4**) after 1 h. The mixture was stirred for 18 h, but a significant increase of the S-alkylated product was not observed. Overall, the results confirm the susceptibility

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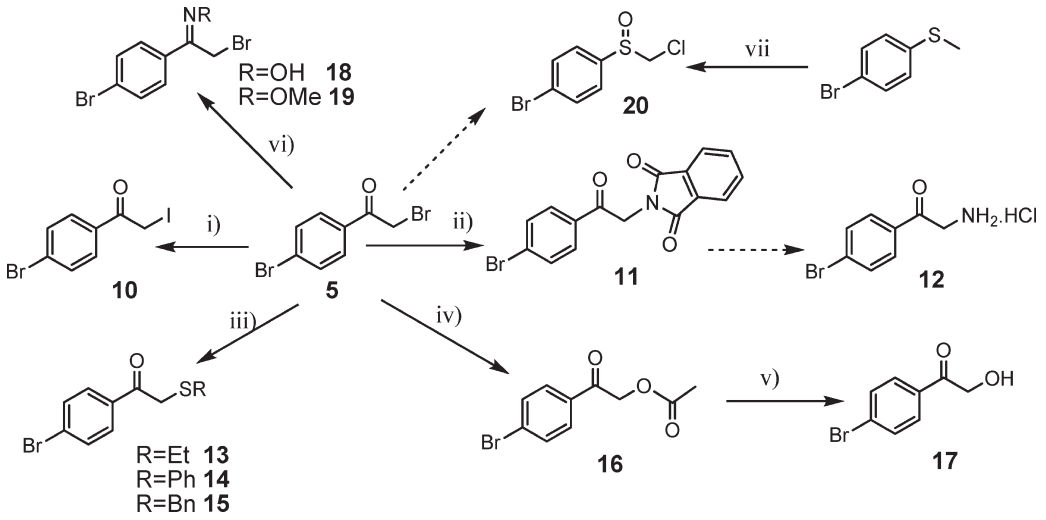
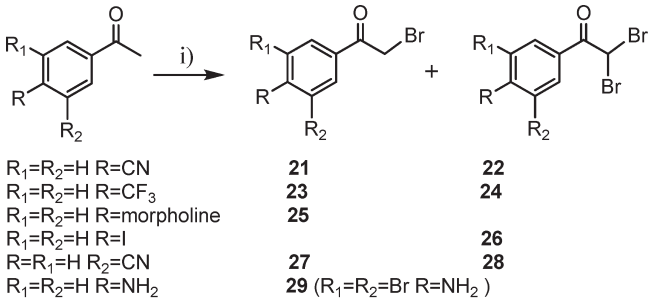
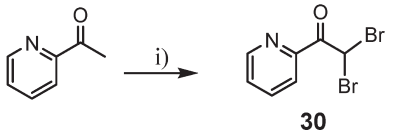
Scheme 1



(i) KI, acetone, Δ; (ii) potassium phthalimide, AcN, Δ; (iii) RSH, K_2CO_3, EtOH; (iv) $(AcO)_2O$, NaAcO, AcOH, Δ; (v) CAL-B, ^{t}BuOH:citrate-phosphate buffer (9:1); (vi) $NH_2OH \cdot HCl$ or $NH_2OMe \cdot HCl$, EtOH, pyr; (vii) SO_2Cl_2, $AgNO_3$, AcN.

Scheme 2.



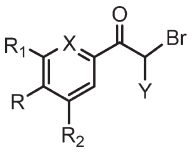


(i) Br_2, $CHCl_3$, 0 °C.

of phenylhalomethylketones to react with thiol groups and the enhanced reactivity upon addition of a suitable base, which might presumably enhance the nucleophilicity of the sulfur atom.

Turning Reversible Inhibitors into Irreversible Inhibition. To confirm the implication of Cys199 in the inactivation of GSK3 by incubation with phenylhalomethylketones, we checked the possibility of turning reversible inhibitors into irreversible ones upon addition of the HMK unit in a proper position that facilitates the reaction with the thiol group of Cys199. To this end, three structurally distinct compounds were selected taking advantage of the known binding mode to GSK-3 from the X-ray crystallographic structures deposited in the Protein Data Bank: an analogue of ATP (PDB entry 1J1B), and two reversible inhibitors (PDB entries 3F88 and 1R0E). Accordingly, three classes of HMK-containing compounds—derivatives of adenine (**31**, **32**), benzimidazole (**35**, **36**), and maleimide (**37**−**40**)—were considered for chemical modification with Cys199 (Figure 1).

Table 2. GSK-3 Inhibitory Activity of Bromoacetyl Aryl Derivatives



No.	R	R_1	R_2	X	Y	IC_{50} (μM)
5	Br	H	H	CH	H	0.5
21	CN	H	H	CH	H	1.5
22	CN	H	H	CH	Br	1.5
23	CF_3	H	H	CH	H	5.0
24	CF_3	H	H	CH	Br	5.0
25		H	H	CH	H	6.0
26	I	H	H	CH	Br	2.0
27	H	H	CN	CH	H	1.0
28	H	H	CN	CH	Br	1.0
29	NH_2	Br	Br	CH	Br	0.5
30	H	H	H	N	Br	2.5

The feasibility of this strategy was first explored by means of docking calculations, which were performed using *rDock* (see Experimental Section. Molecular Modeling). The reliability of *rDock* was assessed by docking a set of known GSK-3 inhibitors taking advantage of the X-ray crystallographic structures of their complexes with the enzyme (PDB entries 1J1B, 3F88, 1R0E,

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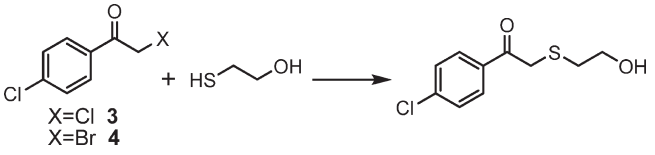
1Q5K, 1UV5, and 2O5K). It is noteworthy that the unrestrained docking protocol was able to predict satisfactorily the X-ray orientation of the compounds in five out of the six cases, as the experimental binding mode was identified as the first or second ranked pose with root-mean-square deviations less than 1.8 Å (see Table S1 in Supporting Information). The X-ray binding mode of the ATP analogue had a higher rank (PDB entry 1J1B), as expected from the larger flexibility of the phosphodiester chain (see Table S1). Inspection of the predicted poses derived for docking calculations for compounds **32**, **36**, and **40** revealed the existence of ligand orientations in the binding site that yielded the HMK moiety suitably positioned for chemical modification by Cys199. This is illustrated for **36** in Figure 2 (see also Figure S1 for compounds **32** and **40**), which shows the second best pose of the compound, which places the C_α atom of the HMK moiety at around 3.6 Å from the sulfur atom in Cys199).

N-Acylation of adenine was achieved by treatment of acetic anhydride and pyridine in DMSO[23] at room temperature overnight (Scheme 3). The precipitate formed was filtered and washed with cold pyridine and ether to achieve the desired product **31** (51% yield). No bromoacetyl derivative of compound **31** was obtained after treatment of this compound with bromine either at low temperature or reflux. In order to synthesize the haloacetyl derivative **32** of compound **31**, reaction was carried out employing adenine, pyridine, and chloroacetic anhydride in THF.

The synthesis of benzimidazole compounds is described in Scheme 4.[24] 1-Bromo-2-nitro-4-acetyl-benzene was treated with

Table 3. Formation of S-Alkylated Products in the Reaction of Compounds 3 and 4 with Mercaptoethanol



	1 h		18 h	
reaction conditions	SM[a]	FP[b]	SM[a]	FP[b]
3:SHEtOH 1:1	88%	11%	24%	74%
3:SHEtOH 1:10	81%	19%	20%	78%
3:SHEtOH:Et_3N 1:1:1	33%	65%	34%	65%
4:SHEtOH 1:1	80%	20%	50%	50%
4:SHEtOH 1:10	47%	53%	----	100%
4:SHEtOH:Et_3N 1:1:1	33%	66%	25%	75%

[a] SM: starting material (compound **3** or **4**). [b] FP: Final product (S-adduct).

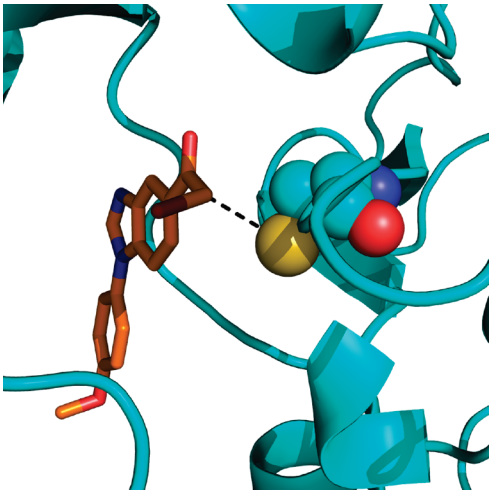


Figure 2. Docked pose for compound **36** with the halomethyketone moitey suitably positioned for chemical modification by Cys199 (shown in spheres; distance from S atom in Cys199 to the C_α atom around or less than 4 Å).

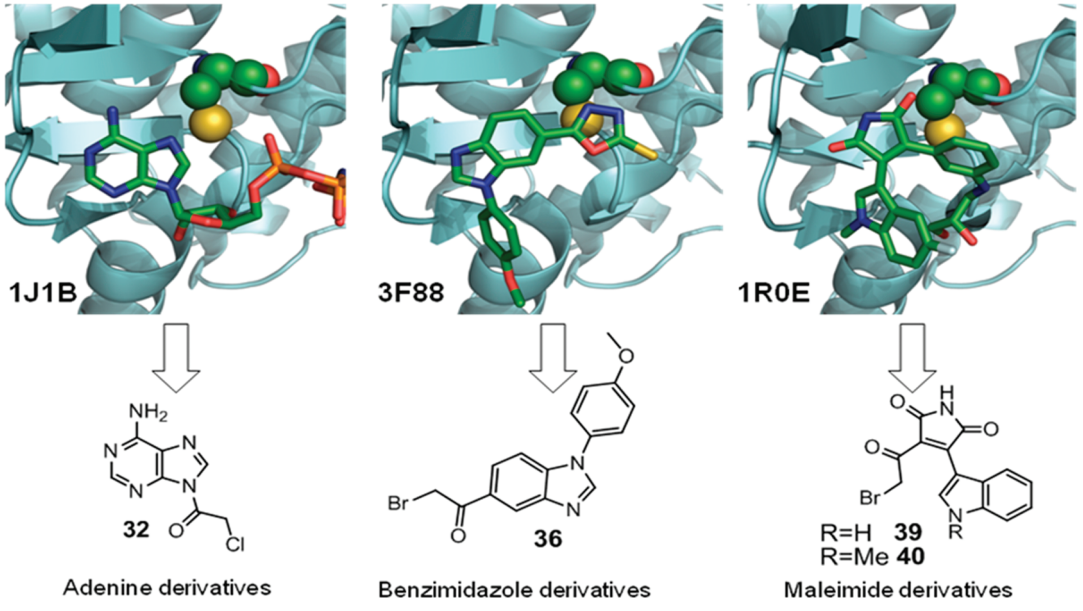


Figure 1. (Top) Representation of the binding mode of an ATP analogue (1J1B), and two reversible inhibitors (3F88 and 1R0E; shown in sticks) in the ATP-binding site of GSK-3. Cys199 is shown in spheres. (Bottom) Structure-based design of novel compounds with potential GSK-3 inhibitory activity.

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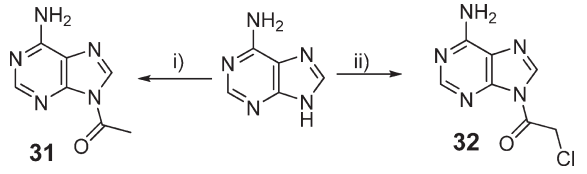
4-methoxyaniline and sodium acetate in DMF to obtain compound **33** in good yield (73%) after recrystallization. The nitro moiety was reduced under catalytic hydrogenation conditions to yield quantitatively derivative **34**. Final cyclization to synthesize benzimidazole **35** was achieved employing formic acid and HCl 4 N solution. The desired halomethylketone **36** was obtained by reaction of the benzimidazole **35** with bromine in acetic acid under reflux.

Finally, the direct formation of the maleimide moiety was chosen for synthesis of maleimide derivatives (Scheme 5).[25] Because commercial indole products required to synthesize those compounds do not contain a fluorine atom in their structure, we eliminated the fluorine atom from the structure originally proposed. The reaction conditions were carefully optimized to avoid the formation of the major byproduct indoleglyoxylic acid. Thus, methyl 2-indoleglyoxylate or 2-(1-methyl-indol)-glyoxylate was added to a solution of potassium *tert*-butoxide and acetamide in tetrahydrofuran at −60 °C. Under these conditions, compound **37** was obtained with good yield (74%), whereas the methyl indole compound **38** reached 56% yield. These compounds were treated with bromine in acetic acid under reflux to yield HMK compounds **39** and **40**, respectively.

Scheme 3



(i) Acetic anhydride, DMSO, rt; (ii) Chloroacetic anhydride, THF, rt.

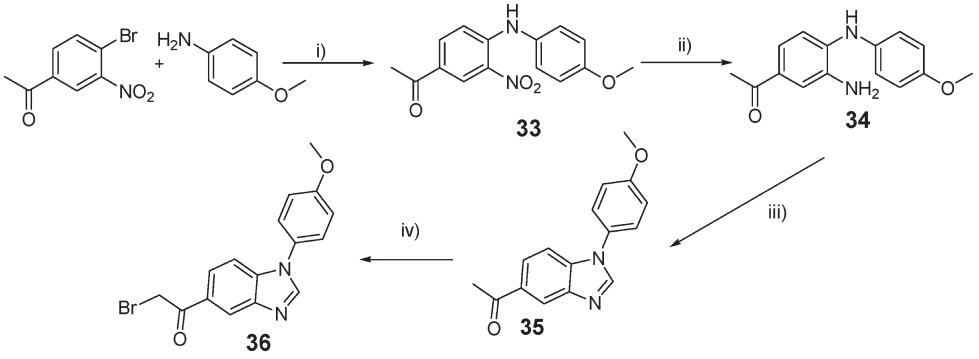
All the newly prepared acetyl and halomethylketo heterocycles **31**, **32**, and **35**−**40** were evaluated as GSK-3 inhibitors using a recently well described luminescent technique[26] as a safer nonradioactive assay (see Table 4). The four HMK-containing compounds (**32**, **36**, **39**, and **40**) are more potent that their corresponding acetyl derivatives (**31**, **35**, **37**, and **38**, respectively), showing in one case an IC_{50} value in the nanomolar range.[27] More importantly, the inhibitory activity determined at different preincubation times confirmed the switch from reversible to irreversible inhibition (Figure 3). Thus, the inhibition of the acetyl-containing compounds **37** and **38** remained unaltered at different preincubation times, mimicking the behavior of alsterpaullone, a known reversible inhibitor. In contrast, the enzyme inhibition increased with preincubation time for the thiophene irreversible inhibitor **HMK-32**[17] and for the HMK-containing compounds **36**, **39**, and **40**. Overall, these findings are in agreement with our working hypothesis, which provides new clues for the specific design of irreversible GSK-3 inhibitors.

The covalent binding of compound **40** to GSK-3 was confirmed biophysically by MALDI-TOF analyses.[28] Compounds **38** and **40** were added in two different experiments to the GSK-3 enzyme under the same conditions as that used for

Table 4. GSK-3 Inhibition Values and Binding Mode of Computer Aided-Designed New Inhibitors

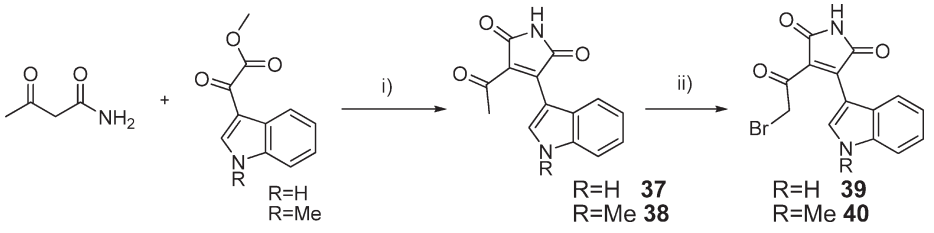
no.	IC_{50} (μM)	binding mode	no.	IC_{50} (μM)	binding mode
31	>50	----	**35**	>10	----
32	13.4	irreversible	**36**	0.58 ± 0.07	irreversible
37	4.47 ± 0.35	reversible	**38**	0.89 ± 0.19	reversible
39	0.047 ± 0.007	irreversible	**40**	0.005 ± 0.001	irreversible

Scheme 4



(i) Sodium acetate, DMF, 100 °C; (ii) H_2, Pd(C), 20 psi THF, rt; (iii) Formic acid, HCl; (iv) Br_2, acetic acid, Δ.

Scheme 5



(i) ^{t}BuOK, THF, −60 °C to 20 °C; (ii) Br_2, acetic acid, Δ.

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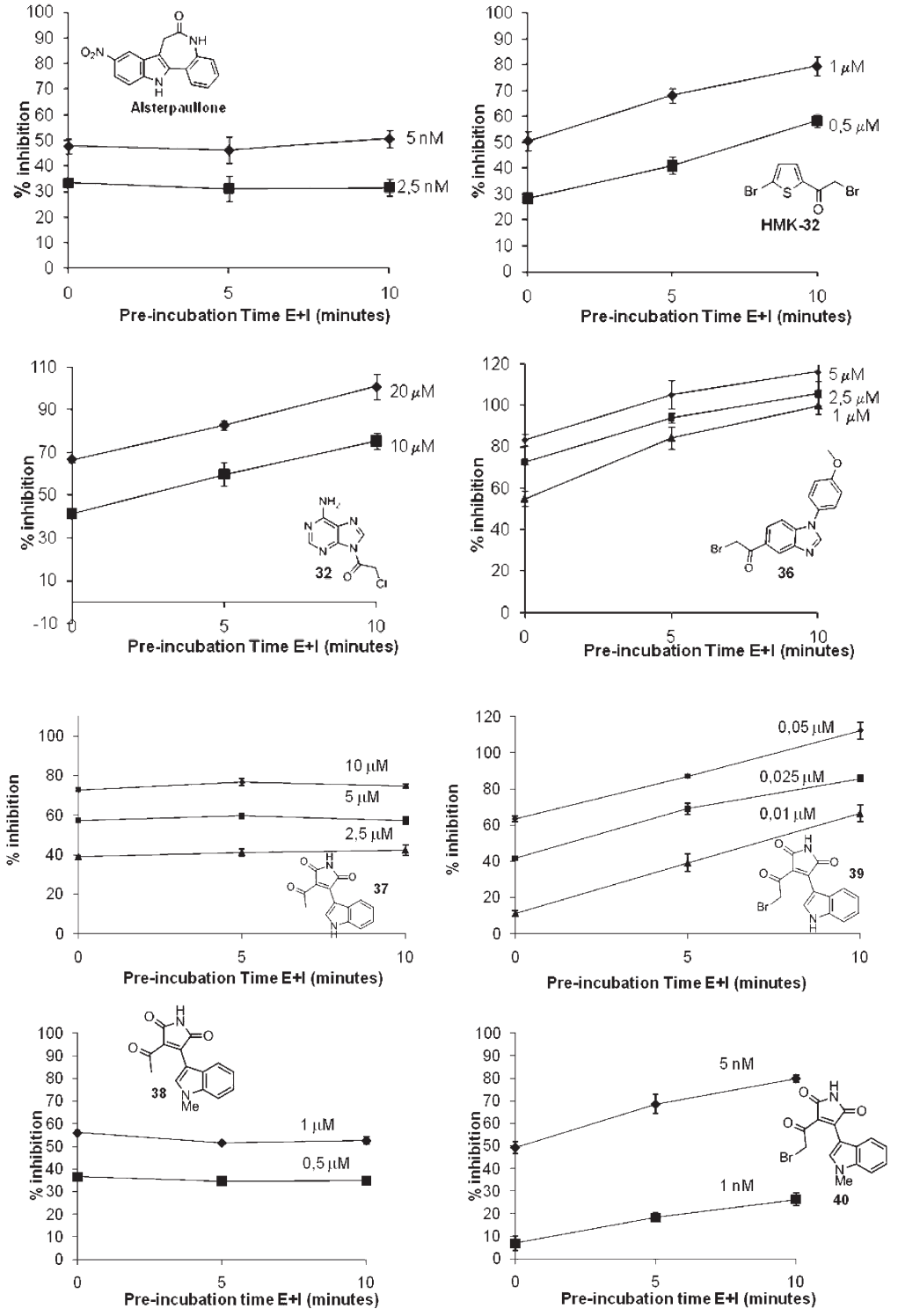

Figure 3. Time-dependent GSK-3 inhibition of controls (alsterpaullone and HMK-32) and the newly synthetized protein kinase inhibitors.

IC_{50} measurements. Mass analysis revealed that, when the reversible inhibitor **38** was used, only the mass corresponding to GSK-3 (48 794 Da) was detected, whereas the use of an irreversible inhibitor (compound **40**) showed a mass peak of 49 036 Da confirming its covalent irreversible binding to the enzyme (Figure 4).

Cellular Effects of Phenylhalomethylketones on GSK-3 Inhibition. All the preceding results suggest that phenylhalomethylketones may be versatile pharmacological tools to explore GSK-3-mediated cellular processes in physiological or pathological conditions. As GSK-3 is involved in tau phosphorylation in transfected cells and *in vivo*,[29] we further explored their ability to interfere with the GSK-3 mediated tau phosphorylation in cells using primary granule cerebellar neurons (GCNs), which also allow us to evaluate the cell permeability of the new inhibitors.

The effect on tau phosphorylation was determined by Western blotting, and detection was carried out using specific

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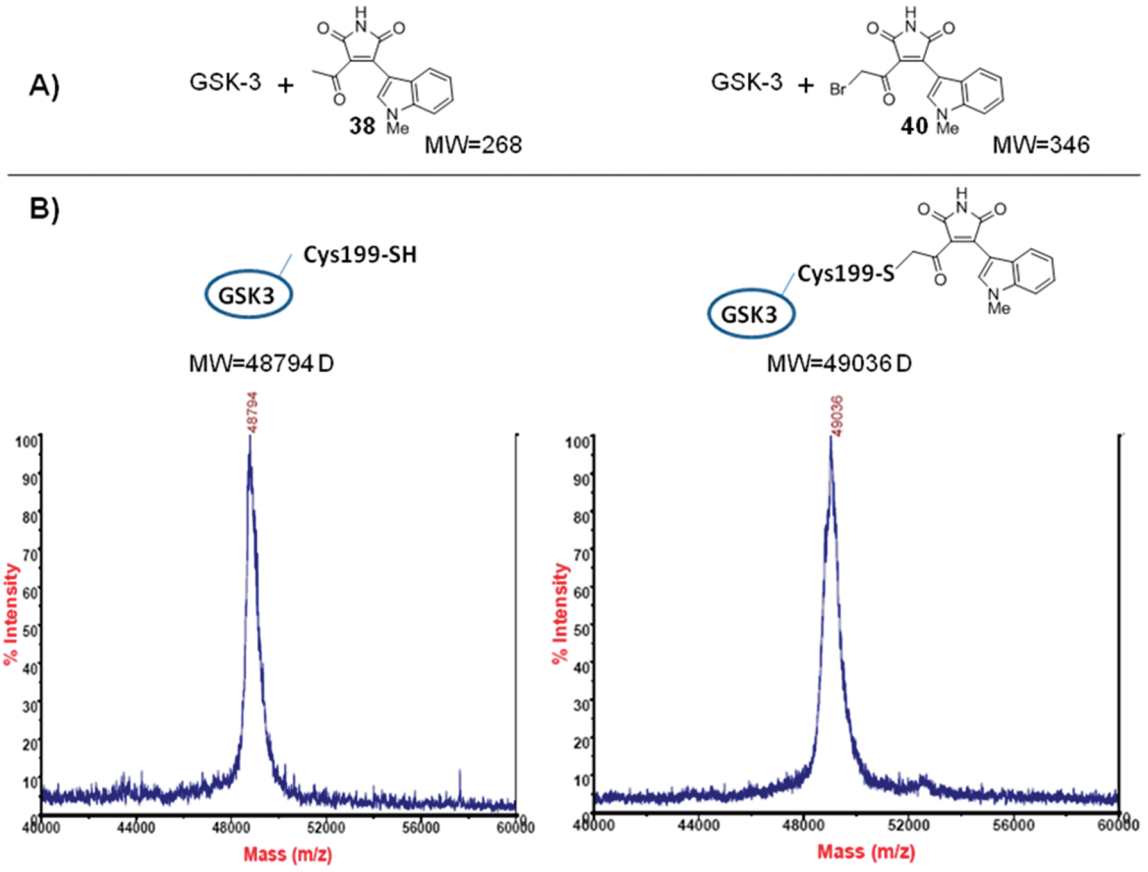

Figure 4. Maldi-TOF spectra. (A) Reversible inhibitor **38** (MW = 268) with GSK-3 (MW = 48 794) showed a 48 794 mass peak. (B) Irreversible inhibitor **40** (MW = 346) with GSK-3 showed a 49 036 mass peak corresponding to the covalent bond S−C formed between the GSK-3 enzyme and the inhibitor **40**.

antibodies: Tau-1 for tau inmunoreactivity, and PHF-1 as tau phosphospecific Ser396 reagent. This last epitope is specifically phosphorylated by GSK-3 on tau protein.[30] Lithium chloride, the first reported GSK-3 inhibitor, was used as standard reference and was added as positive control to the medium at 20 mM. The bands were quantified by densitometric analysis. Following this procedure, data showed that the addition of phenylhalomethylketone **6** or **8** reduced (PHF-1) or increased (Tau-1) the tau-immunoreactivity, thus mimicking the effect observed when lithium chloride was used as a GSK-3 inhibitor (IC_{50} = 1.5 mM) (Figure 5).

Kinase and Neurotransmitter Binding Profiles of Phenylhalomethyl Ketones. To examine the PK selectivity of phenylhalomethylketones for GSK-3, compounds **3**, **5**, and **6** were tested as inhibitors of serine/threonine cAMP-dependent protein kinase (PKA). All of them remained inactive at the highest concentration (100 μM) used. Furthermore, the same set of compounds was evaluated against a panel of eight different serine/threonine and tyrosine kinases (CaM-K II, MAP-K, EGFR-K, IR-K MeK1-K, Abl-K, PKp56, and Src), which are involved in very different signaling pathways not only in neurodegeneration (Cam-KII and MAP-K), but also in other physiological processes and diseases, such as insulin signal pathway (IR-K) or cancer (Abl-K, EGFR-K, MeK1-K, and Src). The inhibitory assays (performed using a 10 μM concentration for the different compounds) showed that, in general, HMKs did not exhibit a significant inhibitory effect on the whole set of kinases (Table 5).

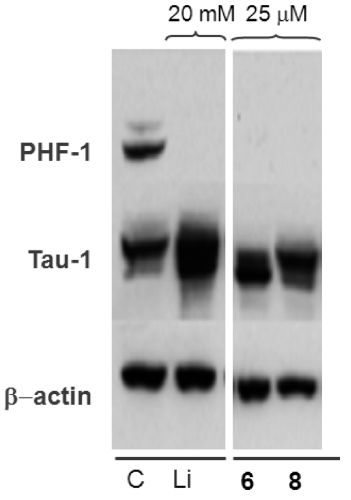

Figure 5. Representative Western blot of soluble extracts from Granule cerebellar neurons (GCNs), obtained after 16 h in the presence of lithium chloride (Li) or different phenylhalomethylketones (**6** and **8**). C represents cell extracts from control GCNs. Identical samples were incubated with antitau-1, anti PHF-1, or anti-β-actin, which was used as a loading control. In each experiment, PHF-1 was normalized with respect to the amount of actin present in each cell extract.

Only the proto-oncogenic Src, which is elevated in some human tumors, is smoothly inhibited by the three selected compounds.

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Table 5. Inhibitory Activity (% Inhibition)[a] Showed by Selected Phenylhalomethylketones for Several Kinases

compound (10 μM)	Abl-K	Cam-KII	EGFR-K	IR-K	MAP-K	MEK-1 K	PK p56	Src-K
3	–	16	–	–	–	–	–	19
5	–	–	18	–	–	–	29	38
6	–	15	10	–	–	–	38	69

[a] The symbol – indicates an inhibitory activity of less than 10%.

Table 6. Inhibitory Activity (% Inhibition)[a] Exhibited by Selected Phenylhalomethylketones for Neurotransmitter Receptors

compound (10 μM)	α2	*h*D2	*h*D3	AMPA	NMDA	*h*M	N (αBGT insensitive)	N (αBGT sensitive)	5-HT
3	–	–	–	–	15	–	–	–	–
5	–	–	–	–	–	–	16	–	–
6	10	–	–	–	–	13	–	–	–

[a] The symbol – indicates inhibitory activity of less than 10%.

Furthermore, it is also important for pathway analysis *in vivo* and in cellular systems to evaluate the potential off-target effects of these new pharmacological tools. This is of particular relevance in phenylhalomethylketones due to the presence of a potential reactive group in their chemical structure, which might react with different neurotransmitter receptors, thus masking a neat GSK-3 response. In order to determine the potential off-target effects of these compounds, we evaluated the *in vitro* binding to different neurotransmitter receptors: α-adrenergic (α_2), dopaminergic (hD2 and hD3), glutamatergic (AMPA and NMDA), muscarinic (hM), nicotinic (bungarotoxin sensitive and insensitive), and serotoninergic (5-HT) receptors. Results showed that compounds **3**, **5**, and **6** (at 10 μM concentration) did not bind to any of these neurotransmitter receptors (Table 6).

CONCLUSIONS

GSK-3 is a therapeutically valuable kinase that is very promising for severe unmet diseases such as AD. To the best of our knowledge, only a small molecule acting as GSK-3 inhibitor, the TDZD compound called Tideglusib, has reached phase IIb clinical trials for the treatment of AD. Additionally, lithium is used in the clinic as the first option for treatment of bipolar disorders, and it is known to inhibit GSK-3 among other mechanisms of action.

The irreversible inhibition of GSK-3 reported for thienylhalomethylketones was suggested to involve covalent modification of Cys199,[17] which is located in the ATP-binding site. Modification of Cys199 might then be a new strategy for the design of specific GSK-3 inhibitors. At this point, the series of phenylhalomethylketone derivatives examined confirms unequivocally the key role of the HMK moiety on GSK-3 inhibition. Furthermore, the implication of Cys199 is supported by the structure-based design of novel compounds characterized by reversible inhibition, which turn out to be irreversible inhibitors upon addition of an HMK unit to their chemical structure.

Overall, chemical modification of Cys199 represents a new mechanism of action that opens new avenues for the development of potent, selective GSK-3 inhibitors. In this context, it is worth highlighting that phenylhalomethylketones (i) act as cell-permeable inhibitors, (ii) decrease tau phosphorylation in cell cultures, (iii) are rather selective against a panel of protein kinases, and (iv) do not bind to different neurotransmitter receptors. Thus, they may be considered useful pharmacological tools to explore physiological and pathological cellular signaling pathways in which GSK-3 is involved.

These findings are especially relevant, as an emerging aspect in the effectiveness of protein kinases inhibitors *in vivo* is the ability to compete with cellular concentrations of ATP (typically about 1–10 mM). For epidermal growth factor receptor kinase (EGFR-K), such problems have been overcome with compound HKI-272, which binds covalently to a cysteine residue.[31] A similar strategy has been recently reported for the irreversible inhibition of HCV NS3/4A viral protease by targeting a noncatalytic cysteine residue.[32] In this context, the strategy explored here, which relies on the key role of Cys 199 in GSK-3 inhibition, might then represent a promising strategy for the development of novel GSK-3 inhibitors for the treatment of severe human diseases.

EXPERIMENTAL SECTION

Chemistry. *General.* Substrates were either purchased from commercial sources or used without further purification. Melting points were determined with a Reichert-Jung Thermovar apparatus and a Mettler Toledo MP70 apparatus and are uncorrected. Flash column chromatography was carried out at medium pressure using silica gel (E. Merck, grade 60, particle size 0.040–0.063 mm, 230–240 mesh ASTM) with the indicated solvent as eluent. Compounds were detected with UV light (254 nm). ^{1}H NMR spectra were obtained on a Gemini-200 spectrometer working at 200 MHz and on a Bruker AVANCE-300 spectrometer working at 300 MHz. Typical spectral parameters: spectral width 10 ppm, pulse width 9 μs (57°), data size 32 K. ^{13}C NMR experiments were carried out on the Varian Gemini-200 spectrometer operating at 50 MHz and on the Bruker AVANCE-300 spectrometer operating at 75 MHz. The acquisiton parameters: spectral width 16 kHz, acquisition time 0.99 s, pulse width 9 μs (57°), data size 32 K. Chemical shifts are reported in values (ppm) relative to internal Me_4Si and *J* values are reported in Hertz. Elemental analyses were performed by the analytical department at CENQUIOR (CSIC), and the results obtained were within ±0.4% of the theoretical values. The combustion analyses are provided in the Supporting Information. Mass spectra were obtained by electronic impact in a Hewlett-Packard 5973 spectrophotometer. All compounds are >95% pure by HPLC analyses. HPLC analyses for compounds 10–30 were performed on a Waters 6000 instrument, with UV detector (214–254 nm), using different columns; μ Bondapack C18, 10 μm, 125 Å, (300 × 3.9 mm) and Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm). Acetonitrile/H_2O [(0.05% H_3PO_4 + 0.04% Et_3N)] 50/50 was used as mobile phase. For the chemical reactivity of HMKs versus thiol groups and HPLC analyses for compounds **31**–**40**, experiments were performed on Alliance Waters 2690 equipment with a UV detector photodiode array Waters 2996 with MS detector MicromassZQ

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(Waters) positive electrospray, using the Sunfire column C18, 3.5 μm (50 × 4.6 mm) and acetonitrile (0.08% formic acid) and MilliQ water (0.1% formic acid) as mobile phase in a 10 min run from 10% to 100% acetonitrile gradient at a flow rate of 0.25 mL/min.

MALDI-MS measurements were performed on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser (λ = 337 nm, 3 ns pulse width, and 3 Hz frequency) and a delayed extraction ion source. Ions generated by the laser desorption were introduced into a time-of-flight analyzer (1.3 m flight path) with an acceleration voltage of 25 kV, a 88% grid voltage, a 0.3% ion guide wire voltage, and a delay time of 500 ns in the linear positive ion mode.

Mass spectra were obtained over the m/z range 30 000−125 000 μ. External mass calibration was applied using the monoisotopic [M+H]+ value of Enolase. Sinapinic acid (>99%; Fluka, Buchs, Switzerland) at 10 mg mL^{-1} in 0.3% trifluoroacetic acid/acetonitrile, 70:30 (v/v) was used as matrix. Samples were mixed with the matrix at a ratio of approximately 1:1, and 1 μL of this solution was spotted onto a flat stainless steel sample plate and dried in air.

*1-(4-Bromophenyl)-2-iodoethanone (**10**).*[33] A mixture of 2-bromo-1-(4-bromophenyl)ethanone (**5**) (2.0 g, 7.20 mmol) and potassium iodide (8.40 g, 50 mmol) in acetone (50 mL) was stirred and refluxed for 3 h. The solvent was evaporated, and CH_2Cl_2 (100 mL) and H_2O (100 mL) were added to the reaction mixture. The organic layer was dried over $MgSO_4$ and the solvent was evaporated. Compound **10** was purified by silica gel chromatography (hexane/ethyl acetate 6:1), 1.91 g (82%), colorless solid, mp 95−96 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.83 (d, *J*= 8.3 Hz, 2H), 7.60 (d, *J*= 8.3 Hz, 2H), 4.30 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 190.7, 131.6, 129.5, 127.8, 123.7, 24.3. m/z (EI): 326, 324, (M^+, 12, 13%), 185, 183 (M-CH_2I, 97, 100%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 99%, r.t = 14.15 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*2-(2-(4-Bromophenyl)-2-oxoethyl)isoindoline-1,3-dione (**11**).*[34] A mixture of 2-bromo-1-(4-bromophenyl)ethanone (**5**) (0.3 g, 1.08 mmol) and potassium phthalimide (0.21 g, 1.13 mmol) in acetonitrile (50 mL) was stirred and refluxed for 2 h. The solvent was evaporated, and CH_2Cl_2 (100 mL) and H_2O (100 mL) were added to the mixture. The organic layer was washed with a 0.2 N sodium hydroxide solution (50 mL) and water (50 mL). Then the organic layer was dried over $MgSO_4$ and the solvent was evaporated. Compound **11** was purified by silica gel chromatography (hexane:ethyl acetate 3:1), 0.28 g (76%), colorless solid, mp 223−225 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.91 (m, 2H), 7.87 (d, *J*= 8.4 Hz, 2H), 7.76 (m, 2H), 7.67 (d, *J*= 8.4 Hz, 2H), 5.01 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 190.1, 167.8, 134.2, 132.2, 129.6, 123.5, 133.1, 132.7, 132.1, 129.4, 44.0. m/z (EI): 345, 343 (M^+, 21, 21%), 185, 183 (M-CH_2 phthalimide, 98, 100%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 99%, r.t = 3.66 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*2-Amino-4′-bromoacetophenone hydrochloride (**12**).* Commercially available from Sigma Aldrich.

*General Procedure for the Synthesis of Sulfur Derivatives (Compounds **13**−**15**).* A mixture of 2-bromo-1-(4-bromophenyl)ethanone (**5**), potassium carbonate, and the corresponding thiol (ethanethiol, thiophenol, and benzylthiol) in ethanol (50 mL) was stirred and refluxed for 2 h. The mixture was filtered, and CH_2Cl_2 (75 mL) and water (75 mL) were added to the solution. The organic layer was washed with water (2 × 75 mL). Then the organic layer was dried over $MgSO_4$ and the solvent was evaporated. The compounds were purified by silica gel chromatography.

*1-(4-Bromophenyl)-2-(ethylthio)ethanone (**13**).*[35] 2-Bromo-1-(4-bromophenyl) ethanone (**5**) (0.5 g, 1.80 mmol), potassium carbonate (0.25 g, 1.80 mmol), ethanethiol (0.11 g, 1.80 mmol). Purified by silica gel chromatography (hexane:ethyl acetate 4:1), 0.20 g (45%), colorless solid, mp 64−65 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.74 (d, *J* = 8.7 Hz, 2H), 7.50 (d, *J* = 8.7 Hz, 2H), 3.65 (s, 2H), 2.46 (q, *J*= 14.6, 7.3 Hz, 3H), 1.16 (t, *J*= 7.3 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 194.3, 134.7, 132.8, 131.2, 129.4, 37.5, 27.2, 14.9. m/z (EI): 260, 258 (M^+, 10, 9%), 185, 183 (M-$SCH_2CH_2CH_3$, 100, 99%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 99%, r.t = 5.85 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*1-(4-Bromophenyl)-2-(phenylthio)ethanone (**14**).*[36] 2-Bromo-1-(4-bromophenyl) ethanone (**5**) (0.5 g, 1.80 mmol), potassium carbonate (0.25 g, 1.80 mmol), benzenethiol (0.20 g, 1.80 mmol). Purified by silica gel chromatography (hexane:ethyl acetate 4:1), 0.29 g (53%), colorless solid, mp 60−61 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.68 (d, *J*= 8.7 Hz, 2H), 7.49 (d, *J*= 8.7 Hz, 2H), 7.24−7.20 (m, 5H), 4.10 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.8, 134.0, 133.7, 130.4, 130.1, 129.9, 128.8, 128.4, 127.0, 40.7. m/z (EI): 308, 306 (M^+, 55, 52%), 185, 183 (M-SCH_2Ph, 100, 98%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 99%, r.t = 8.15 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*2-(Benzylthio)-1-(4-bromophenyl)ethanone (**15**).*[37] 2-Bromo-1-(4-bromophenyl) ethanone (**5**) (0.5 g, 1.80 mmol), potassium carbonate (0.25 g, 1.80 mmol), benzylthiol (0.22 g, 1.80 mmol). Purified by silica gel chromatography (hexane:ethyl acetate 4:1), 0.32 g (55%), colorless solid, mp 158−159 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.68 (d, *J*= 8.7 Hz, 2H), 7.49 (d, *J*= 8.7 Hz, 2H), 7.24−7.20 (m, 5H), 3.63 (s, 2H), 3.52 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.3, 136.1, 133.0, 130.9, 129.2, 128.3, 127.6, 126.4, 126.3, 35.1, 34.7. m/z (EI): 322, 320 (M^+, 17, 17%), 200, 198 (M-SCH_2Ph, 85, 83%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 99%, r.t = 9.76 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*2-(4-Bromophenyl)-2-oxoethyl acetate (**16**).*[38] To a solution of 2-bromo-1-(4-bromophenyl)ethanone (**5**) (0.5 g, 1.80 mmol) in acetic acid (50 mL) was added sodium acetate (0.44 g, 5.40 mmol) and acetic anhydride (0.55 g, 5.40 mmol). The mixture was stirred and refluxed for 5 h. CH_2Cl_2 (100 mL) and H_2O (100 mL) were added to the reaction mixture. The organic layer was washed with H_2O (2 × 100 mL), a saturated solution of $NaHCO_3$ (100 mL), and a saturated solution of NaCl (100 mL). The organic layer was dried over $MgSO_4$ and the solvent was evaporated. Compound **16** was purified by silica gel chromatography (hexane:ethyl acetate 7:1), 0.41 g (87%), colorless solid, mp 84−85 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.67 (d, *J* = 8.6 Hz, 2H), 7.35 (d, *J* = 8.6 Hz, 2H), 5.18 (s, 2H), 2.12 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 190.3, 169.3, 131.8, 131.2, 128.2, 128.1, 64.8, 19.5. m/z (EI): 260, 258 (M^+, 15, 15%), 183, 181, 179 (M-$C_3H_5O_2$, 25, 89, 100%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 100%, r.t = 6.41 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*1-(4-Bromophenyl)-2-hydroxyethanone (**17**).*[39] Enzymatic deacylation of **16**, was done employing different lipases (PPL (*Pancreatic Porcine Lipase*), PSL (*Pseudomonas cepacia Lipase*), MML (*Mucor miehi Lipase*), and CAL-B (*Candida Antartica Lipase-B*) under the same reaction conditions (citrate-phosphate 9:1 buffer pH 7, *tert*-butanol, 45 °C, 24 h). No product was obtained using MML, while partial hydrolysis was done when lipases PPL and PSL were employed. However, the use of CAL-B gave exclusively compound **17** with an 85% yield. Accordingly, a mixture of 2-(4-bromophenyl)-2-oxoethyl acetate (**16**) (20 mM) and CAL-B (20 mM) in *tert*-butanol:citrate phosphate buffer (pH = 7) 9:1 (85 mL) was orbitally stirred and refluxed for 24 h. The reaction was filtered and ethyl acetate (75 mL) and H_2O (50 mL) were added to the solution. The organic layer was dried over $MgSO_4$ and the solvent was evaporated. Compound **17** was purified by silica gel chromatography (hexane:ethyl acetate 1:1), 0.36 g (85%), colorless solid, mp 103−104 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.80 (d, *J* = 8.6 Hz, 2H), 7.65 (d, *J* = 8.6 Hz, 2H), 4.85 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 197.8, 132.9, 132.4, 130.1, 129.5, 65.7. m/z (EI): 216, 214 (M^+, 11, 11%), 185, 183, (M-CH_3O, 100, 100%). HPLC: Column Symmetry

C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 99%, r.t = 3.81 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*General Procedure for Oxime (**18**) and Methyl Oxime Synthesis (**19**).* To a mixture of hydroxylamine hydrochloride or methylhydroxylamine hydrochloride and pyridine in ethanol (50 mL) was added 2-bromo-1-(4-bromophenyl)ethanone (**5**). The mixture was stirred and refluxed for 4 h. The solvent was evaporated and cold water (25 mL) was added to the mixture. The mixture is left at 0 °C for 12 h and the precipitate formed was filtered. The compounds were purified by silica gel chromatography.

*(E,Z)-2-Bromo-1-(4-bromophenyl)ethanone Oxime (**18**).*[40] Hydroxylamine hydrochloride (0.26 g, 3.81 mmol), pyridine (0.31 g, 3.81 mmol), and 2-bromo-1-(4-bromophenyl)ethanone (**5**) (1.0 g, 3.59 mmol). Purified silica gel column chromatography (hexane:ethyl acetate 6:1), 0.82 g (78%), colorless solid, mp 115−116 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 11.8, 11.7 (s, 1H), 7.52−7.41 (m, 4H), 4.48, 4.35 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.0, 153.9, 132.7, 132.3, 128.2, 124.7, 32.1, 27.2. *m/z* (EI): 295, 293, 291 (M^+, 36, 72, 38%), 102 (M-CH_3Br_2O, 100%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 99%, r.t = 8.15 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*(E,Z)-2-Bromo-1-(4-bromophenyl)ethanone O-Methyl Oxime (**19**).*[41] Methyl hydroxylamine hydrochloride (0.30 g, 3.59 mmol), pyridine (0.28 g, 3.59 mmol), and 2-bromo-1-(4-bromophenyl)ethanone (**5**) (1.0 g, 3.59 mmol). Purified by silica gel chromatography (hexane:ethyl acetate 6:1), 0.56 g (82%), colorless solid, mp 48−49 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.65−7.51 (m, 4H), 4.52, 4.35 (s, 2H), 4.15, 4.05 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 151.2, 132. 4, 131.9, 127.7, 124.1, 63.0, 62.9, 32.2, 32.1. *m/z* (EI): 309, 307, 305 (M^+, 13, 26, 13%), 102 (M-$C_2H_5Br_2O$, 100%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 99%, r.t = 8.10 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*1-Bromo-4-(chloromethylsulfinyl)benzene (**20**).*[42] A solution of sulfuryl chloride (0.33 g, 2.46 mmol) in acetonitrile (5 mL) was added dropwise to a mixture of bromothioanisol (0.50 g, 2.46 mmol) and silver nitrate (0.42 g, 2.46 mmol) in acetonitrile (10 mL) at 0 °C. The mixture was then stirred 1 h at room temperature. CH_2Cl_2 (10 mL) and H_2O (10 mL) were added to the reaction mixture. The organic layer was washed with H_2O (2 × 100 mL), dried over $MgSO_4$, and the solvent was evaporated. Compound **20** was purified by silica gel chromatography (hexane:ethyl acetate 1:1), 0.44 g (71%), colorless solid, mp 91−92 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.71 (d, *J* = 8.7 Hz, 2H), 7.58 (d, *J* = 8.7 Hz, 2H), 4.38 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 142.1, 132.6, 126.4, 93.1, 6.8. *m/z* (EI): 256, 254, 252 (M^+, 4, 16, 12%), 205, 203 (M-CH_2Cl, 100, 100%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 99%, r.t = 4.73 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*General Procedure for Bromoacetyl Benzene (**21**, **23**, **25**, **27**, and **29**), Dibromoacetyl Benzene (**22**, **24**, **26**, **28**), and Pyridine (**30**) Synthesis.* To a solution of acetophenone derivative or 2-acetyl pyridine dissolved in $CHCl_3$ (35 mL) was added dropwise a solution of bromine in $CHCl_3$ (10 mL) at 0 °C. The mixture was stirred 2 h. The solvent was evaporated and to the mixture was added CH_2Cl_2 (50 mL) and a saturated solution of $NaHCO_3$ (50 mL). The organic layer was dried over $MgSO_4$ and the solvent was evaporated. Compounds were purified/separated by silica gel chromatography.

*4-(2-Bromoacetyl)benzonitrile (**21**)*[43] *and 4-(2,2-Dibromoacetyl)-benzonitrile (**22**).*[44] 4-Acetylbenzonitrile (1.0 g, 6.89 mmol), bromine (1.12 g, 7.02 mmol). Mixture of compounds separated by silica gel chromatography (hexane:ethyl acetate 3:1), 0.69 g (45%) (**21**), colorless solid, mp 90−91 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.10 (d, *J* = 8.79 Hz, 2H), 7.82 (d, *J* = 8.79 Hz, 2H), 4.44 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 190.4, 137.3, 133.0, 129.8, 118.0, 117.6, 30.4. *m/z* (EI): 225, 223 (M^+, 4, 4%), 130 (M-$COCH_2Br$, 100%). HPLC: Column μ Bondapack C18, 10 μm, 125 Å, (300 × 3.9 mm), Purity 97%, r.t = 4.41 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50. Compound (**22**). 0.31 g (15%), colorless solid, mp 95−96 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.23 (d, *J* = 8.79 Hz, 2H), 7.83 (d, *J* = 8.79 Hz, 2H), 6.58 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.0, 134.4, 132.9, 130.7, 117.9, 117.8, 39.0. *m/z* (EI): 305, 303, 301 (M^+, 4, 12, 4%), 130 (M-$COCHBr_2$, 100%). HPLC: μ Bondapack C18, 10 μm, 125 Å, (300 × 3.9 mm), Purity 98%, r.t = 4.11 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*2-Bromo-1-(4-(trifluoromethyl)phenyl)ethanone (**23**)*[45] *and 2,2-Dibromo-1-(4-(trifluoromethyl)phenyl)ethanone (**24**).*[46] 1-(4-(Trifluoromethyl)phenyl) ethanone (1.0 g, 5.31 mmol), bromine (1.10 g, 6.90 mmol). Mixture of compounds separated by silica gel chromatography (hexane:ethyl acetate 8:1), 0.18 g (6%) (**23**), colorless solid, mp 55−56 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.12 (d, *J* = 8.06 Hz, 2H), 7.79 (d, *J* = 8.06 Hz, 2H), 4.47 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 190.4, 136.5, 135.0 (q, J_{CF} = 33.09 Hz), 129.3, 125.9, 123.3. (q, J_{CF} = 272.9 Hz), 30.4. *m/z* (EI): 268, 266 (M^+, 6, 6%), 172 (M-$COCH_2Br$, 100%). HPLC: Column μ Bondapack C18, 10 μm, 125 Å, (300 × 3.9 mm), Purity 97%, r.t = 6.53 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50. Compound (**24**). 0.64 g (45%), colorless solid, mp 41−42 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.22 (d, *J* = 8.3 Hz, 2H), 7.81 (d, *J* = 8.3 Hz, 2H), 6.6 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 184.1, 134.7 (q, *J* = 272.9 Hz), 132.7, 129.2, 124.8, 119.5 (q, *J* = 33.09), 38.2. *m/z* (EI): 348, 346, 344 (M^+, 1, 6, 1%), 173 (M-$CHBr_2$, 100%). HPLC: Column μ Bondapack C18, 10 μm, 125 Å, (300 × 3.9 mm), Purity 97%, r.t = 9.96 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*2-Bromo-1-(4-morpholinophenyl)ethanone (**25**).*[47] 1-(4-Morpholinophenyl) ethanone (1.0 g, 4.87 mmol), bromine (1.01 g, 6.33 mmol). Purified by silica gel chromatography (hexane:ethyl acetate 1:1), 0.56 g (42%) (**25**), colorless solid, mp 108−109 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.95 (d, *J* = 4 Hz, 2H), 7.04 (d, *J* = 4 Hz, 2H), 4.60 (s, 2H), 3.83 (m, 4H), 3.65 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 189.9, 154.9, 130.5, 120.3, 113.5, 67.8, 47.6, 26.7. *m/z* (EI): 285, 283 (M^+, 14, 13%), 190 (M-CH_2Br, 100%). HPLC: Column μ Bondapack C18, 10 μm, 125 Å, (300 × 3.9 mm), Purity 99%, r.t = 4.56 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*2,2-Dibromo-1-(4-iodophenyl)ethanone (**26**).*[46] 1-(4-Iodophenyl)ethanone (0.40 g, 1.60 mmol), bromine (0.1 mL, 1.70 mmol). Purified by silica gel chromatography (hexane:ethyl acetate 6:1), 0.31 g (48%) (**26**), colorless solid, mp 77−78 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.82 (d, *J* = 4.2 Hz, 2H), 7.73 (d, *J* = 4.2 Hz, 2H), 6.3 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.3, 138.6, 131.3, 130.4, 103.4, 39.6. *m/z* (EI): 406, 404, 402 (M^+, 9, 17, 9%), 231, (M-$CHBr_2$, 100%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 98%, r.t = 4.51 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*3-(2-Bromoacetyl)benzonitrile (**27**)*[48] *and 3-(2,2-Dibromoacetyl)benzonitrile (**28**).*[49] 3-Acetylbenzonitrile (0.5 g, 3.44 mmol), bromine (0.72 g, 4.48 mmol). Mixture of compounds separated by silica gel chromatography (hexane:ethyl acetate 5:2), 0.10 g (13%) (**27**), colorless solid, mp 70−71 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.21 (t, *J* = 1.5 Hz, 1H), 8.15 (td, *J* = 7.9, 1.5 Hz, 1H), 7.95 (td, *J* = 7.9, 1.5 Hz, 1H), 7.80 (t, *J* = 7.9 Hz, 1H), 4.32 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 189.8, 137.0, 135.1, 133.2, 133.0, 130.2, 117.9, 113.9, 30.1. *m/z* (EI): 225, 223 (M^+, 2, 2%), 130 (M-CH_2Br, 100%). HPLC: Column μ Bondapack C18, 10 μm, 125 Å, (300 × 3.9 mm), Purity 97%, r.t = 3.60 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50. Compound (**28**). 0.38 g (37%), colorless solid, mp 85−86 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.25 (t, *J* = 1.5 Hz, 1H), 8.15 (td, *J* = 7.9, 1.5 Hz, 1H), 7.85 (td, *J* = 7.9, 1.5 Hz, 1H), 7.70 (t, *J* = 7.9 Hz, 1H), 6.40 (s, 1H, CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 184.5, 137.4, 134.1, 133.8, 132.1, 130.2, 117.8, 114.0, 38.9. *m/z* (EI): 305, 303, 301 (M^+, 3, 9, 3%), 130

(M-$COCHBr_2$, 100%). HPLC: Column μ Bondapack C18, 10 μm, 125 Å, (300 × 3.9 mm), Purity 98%, r.t = 4.10 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*1-(4-Amino-3,5-dibromophenyl)-2-bromoethanone (**29**).*[46] 1-(4-Aminophenyl) ethanone (0.5 g, 3.70 mmol), bromine (0.20 mL, 4.06 mmol). Purified by silica gel chromatography (hexane:ethyl acetate 2:1), 0.80 g (58%) (**29**), colorless solid, mp 146−147 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.0 (s, 2H), 5.1 (s, 2H), 4.2 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.5, 147.3, 132.7, 123.3, 106.2, 38.3. *m/z* (EI): 375, 373, 371, 369 (M^+, 10, 31, 31, 10%), 281, 279, 277 (M-$CHBr_2$, 64, 100, 67%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 96%, r.t = 9.98 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*2,2-Dibromo-1-(pyridin-2-yl)ethanone (**30**).*[50] 1-(Pyridin-2-yl)-ethanone (1.0 g, 8.25 mmol), bromine (1.45 g, 9.08 mmol). Purified by silica gel chromatography (hexane:ethyl acetate 6:1), 1.69 g (74%) (**30**), colorless solid, mp 196−197 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.71 (m, 1H), 8.10 (m, 2H), 7.82 (m, 1H), 6.13 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.0, 149.5, 148.1, 138.4, 129.0, 124.0, 45.0. *m/z* (EI): 281, 279, 277 (M^+, 9, 19, 9%), 106 (M-$CHBr_2$, 100%). HPLC: Column Symmetry C18, 5 μm, 100 Å, (150 × 3.9 mm), Purity 98%, r.t = 3.66 min, acetonitrile/H_2O (0.05% H_3PO_4 + 0.04% Et_3N) 50/50.

*1-(6-Amino-9H-purin-9-yl)ethanone (**31**).*[23] To a solution of adenine (1.23 g, 9.14 mmol) in DMSO anhydrous (50 mL) was added a solution of pyridine anhydrous (20 mL) and acetic anhydride (5 mL). The mixture was stirred for 24 h at room temperature and a white precipitate formed. The precipitate was filtered and washed with several portions of cold pyridine and ether, to afford a white solid (629 mg, 51%), mp 363−364 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.61 (s, 1H), 8.28 (s, 1H), 7.52 (s, 2H), 2.88 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 173.97, 162.01, 159.50, 154.36, 144.05, 125.17, 30.41. HPLC: Purity >99%, r.t = 0.55 min. MS (ESI+): *m/z* 178 $[M+H]^+$.

*1-(6-Amino-9H-purin-9-yl)-2-chloroethanone (**32**).* To a solution of adenine (1.00 g, 7.4 mmol) in THF anhydrous (50 mL) was added a mixture of pyridine anhydrous (20 mL) and chloroacetic anhydride (1.39 g, 8.14 mmol). The mixture was stirred for 24 h at room temperature and a white precipitate was formed. The precipitate was filtered and washed with several portions of cold pyridine and cold ether. This solid was purified by column chromatography (methanol:dichloromethane 1:10) to afford a white solid (312 mg, 20%), mp 199−200 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.28 (s, 1H), 11.48 (s, 2H), 8.65 (s, 1H), 8.45 (s, 1H), 4.55 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 166.32, 151.25, 145.74, 144.20, 118.15, 43.40. HPLC: Purity 95%, r.t = 2.87 min. MS (ESI+): *m/z* 212 $[M+H]^+$.

*1-(4-(4-Methoxyphenylamino)-3-nitrophenyl)ethanone (**33**).*[51] To a solution of 1-(4-bromo-3-nitrophenyl)ethanone (1 g, 4.09 mmol) and 4-methoxyaniline (0.504 g, 4.09 mmol) in anhydrous DMF (23 mL) was added sodium acetate (0.504 g, 6.14 mmol). The mixture was heated 2 h at 100 °C and then stirred at room temperature overnight. Ethyl acetate (100 mL) and H_2O (100 mL) was added to the reaction mixture. The organic layer was washed with H_2O (2 × 100 mL), dried over $MgSO_4$, and the solvent was evaporated. The residue was recrystallized from MeOH/H_2O to afford **33** as a red solid (853 mg, 73%), mp 132−133 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.77 (s, 1H), 8.82 (s, 1H), 7.94 (d, *J* = 8.9 Hz, 1H), 7.21 (d, *J* = 8.7 Hz, 2H), 7.10 − 6.83 (m, 3H), 3.86 (s, 3H), 2.58 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 194.7, 158.4, 147.6, 134.4, 131.1, 129.8, 128.4, 127.2, 126.1, 115.6, 115.1, 55.4, 25.92. HPLC: Purity >99%, r.t = 5.09 min. MS (ESI+): *m/z* 287 $[M+H]^+$.

*1-(4-(4-Methoxyphenylamino)-3-aminophenyl)ethanone (**34**).* To a solution of **33** (0.23 g, 0.8 mmol) dissolved in THF (20 mL) was added Pd(C) (23 mg). The mixture was stirred at room temperature overnight at 20 psi. The mixture was filtered and the solvent was evaporated to afford a brown solid. The title compound was used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.43 (m, 1H), 7.40 (s, 1H), 7.37 (m, 1H), 7.26 (m, 1H), 6.96−6.86 (m, 2H), 6.86−6.68 (m, 2H), 5.64 (s, 1H), 3.81 (s, 3H), 2.51 (s, 3H), 1.44 (s, 1H). HPLC: Purity 88%, r.t = 4.15 min. MS (ESI+): *m/z* 257 $[M+H]^+$.

*1-(1-(4-Methoxyphenyl)-1H-benzo[d]imidazol-5-yl)ethanone (**35**).* A stirred solution of **34** (0.93 g, 3.64 mmol) and formic acid (1.58 mL, 42 mmol) in HCl 4 N (25 mL) was refluxed for 3 h. Then dichloromethane (25 mL) was added to the mixture. The organic layer was washed with saturated $NaHCO_3$ solution (3 × 25 mL) and then dried over $MgSO_4$. The solvent was evaporated under reduced pressure to give **35** as a brown solid (803 mg, 83%), mp 139−140 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.46 (d, *J* = 1.1 Hz, 1H), 8.13 (s, 1H), 7.99 (dd, *J* = 8.6, 1.5 Hz, 1H), 7.50−7.42 (m, 1H), 7.40 (d, *J* = 9.0 Hz, 2H), 7.08 (d, *J* = 9.0 Hz, 2H), 3.89 (s, 3H), 2.69 (s, 3H).^{13}C NMR (75 MHz, $CDCl_3$): δ 198.0, 159.9, 144.6, 132.6, 128.7, 126.0, 123.9, 122.4, 115.5, 110.6, 55.9, 26.9. HPLC: Purity 99%, r.t = 4.18 min. MS (ESI+): *m/z* 267 $[M+H]^+$.

*2-Bromo-1-(1-(4-methoxyphenyl)-1H-benzo[d]imidazol-5-yl)-ethanone (**36**).* To a solution of **35** (1.14 g, 4.28 mmol) dissolved in AcOH (25 mL) at reflux was added a solution of bromine (0.23 mL, 4.50 mmol) in AcOH (5 mL). The mixture was refluxed and stirred for 5 h. Dichloromethane (50 mL) and water (50 mL) were added to the mixture. The organic layer was extracted and washed with saturated solutions of $NaHCO_3$ (2 × 50 mL) and NaCl (50 mL). The organic layer was dried over $MgSO_4$ and the solvent was evaporated. The product was purified by flash chromatography (hexane:ethyl acetate 1:1), 162 mg (11%) (**36**), red solid, mp 247−248 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.52 (s, *J* = 1.1 Hz, 1H), 8.18 (s, 1H), 8.03 (dd, *J* = 8.6, 1.6 Hz, 1H), 7.51 (d, *J* = 8.6, 1H), 7.41 (d, *J* = 9.0 Hz, 2H), 7.10 (d, *J* = 9.0 Hz, 2H), 4.57 (s, 2H), 3.91 (s, 3H).^{13}C NMR (75 MHz, $CDCl_3$): δ 191.2, 160.0, 144.9, 126.0, 124.7, 122.9, 115.5, 111.1, 55.9. HPLC: Purity 95%, r.t = 4.70 min. MS (ESI+): *m/z* 345 $[M+H]^+$.

*General Procedure for Maleimide Synthesis (**37**−**38**).* To a solution of acetoacetamide (1 equiv) and ^{t}BuOK (1 M solution in THF) (3.5 equiv) dissolved in THF (20 mL) at −60 °C was added methyl 3-indoleglyoxylate or methyl 2-(1-methyl-1H-indol-3-yl)-2-oxoacetate (1 equiv). The mixture was stirred until room temperature was reached. Then a concentrated HCl solution (11.5 mL), water (30 mL), and dichlorometane (30 mL) were added in the reaction mixture. The organic layer was washed with a saturated solution of $NaHCO_3$ (2 × 30 mL), dried over $MgSO_4$, and evaporated. Products were purified by recrystallization from ethyl acetate/pentane.

*3-Acetyl-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (**37**).* Acetoacetamide (0.372 g, 3.69 mmol), ^{t}BuOK (1 M solution in THF) (12.9 mL, 12.9 mmol), methyl 3-indoleglyoxylate (0.750 g, 3.69 mmol). 0.70 g (74%) (**37**), orange solid, mp 225−226 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.29 (s, 1H), 11.17 (s, 1H), 8.24 (s, 1H), 7.50 (d, *J* = 7.01 Hz 1H), 7.27−7.17 (m, 1H), 7.13 (s, 2H), 2.50−2.46 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 195.4, 171.7, 139.8, 137.5, 135.5, 125.5, 124.9, 123.5, 122.6, 121.7, 113.3, 106.0, 31.7. HPLC/MS: Purity >99%, r.t = 3.76 min. MS (ESI+): *m/z* 255 $[M+H]^+$.

*3-Acetyl-4-(1-methyl-indol-3-yl)-1H-pyrrole-2,5-dione (**38**).* Acetoacetamide (0.325 g, 3.22 mmol), ^{t}BuOK (1 M solution in THF) (11.3 mL, 11.3 mmol), 2-(1-methyl-1H-indol-3-yl)-2-oxoacetate (0.700 g, 3.22 mmol). 0.482 g (56%) (**38**), red solid, mp 224−225 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.19 (s, 1H), 8.27 (s, 1H), 7.58 (d, *J* = 8.1 Hz, 1H), 7.35−7.06 (m, 3H), 3.92 (s, 3H), 2.51 (s, 3H).^{13}C NMR (75 MHz, DMSO-d_6): δ 194.6, 170.9, 138.5, 138.1, 137.3, 125.2, 123.8, 122.8, 122.1, 121.3, 111.0, 104.3, 33.3, 30.9. HPLC/MS: Purity 95%, r.t = 4.06 min. MS (ESI+): *m/z* 269 $[M+H]^+$.

*General Procedure for Maleimide Bromation (**39** and **40**).* Maleimide derivative **37** or **38** (1 equiv) is dissolved in AcOH. Under reflux conditions, bromine (1.05 equiv) is slowly added diluted in AcOH. The mixture is stirred at reflux temperature for 3 h. Then water and AcOEt are added and the mixture is extracted, and the organic layer washed with

several portions of a $NaHCO_3$ saturated solution and then dried and evaporated. The products are purified by recrystallization MeOH/H_2O (**39**) or by flash chromathography (**40**) using a dichloromethane/methanol 80:1 mixture as eluent.

*3-(2-Bromoacetyl)-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (**39**).* Title compound was prepared using the same procedure described for the synthesis of compound **36**. Maleimide **37** (0.670 g, 2.63 mmol), bromine (0.135 mL, 2.63 mmol). The residue was recrystallized from methanol/water to give **39** as a black solid (0.345 g, 39%), mp 260−261 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.54 (s, 1H), 11.34 (s, 1H), 8.39 (m, 1H), 7.54 (d, J = 8.0 Hz, 1H), 7.32−6.98 (m, 3H), 4.75 (s, 2H). ^{13}C NMR (126 MHz, acetone-d_6): δ 186.0, 169.6, 169.4, 142.1, 135.9, 135.7, 124.7, 122.8, 121.0, 112.1, 106.4, 35.0. HPLC/MS: Purity 95%, r.t = 4.35 min. MS (ESI+): m/z 333 $[M+H]^+$.

*3-(2-Bromoacetyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (**40**).* Title compound was prepared using the same procedure described for the synthesis of compound **36**. Maleimide **38** (0.603 g, 2.25 mmol), bromine (0.115 mL, 2.25 mmol). The product was purified by flash chromatography (dichloromethane:methanol 80:1), 120 mg (15%) (**40**), purple solid, mp 252−253 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.46 (s, 1H), 7.38 (m, 4H), 5.31 (s, 1H), 4.64 (s, 2H), 3.94 (s, 3H). ^{13}C NMR (126 MHz, acetone-d_6): δ 185.80, 169.78, 169.55, 141.80, 139.52, 137.66, 125.37, 122.94, 122.88, 121.34, 119.28, 110.46, 105.53, 35.02, 32.71. HPLC: Purity 99%, r.t = 4.47 min. MS (ESI+): m/z 347 $[M+H]^+$.

Chemical Reactivity of HMKs. 100 μL of an acetonitrile solution of HMK **3** or **4** (50 mM) and 100 μL of an acetonitrile solution of 2-mercaptoethanol (50 mM) were added to a mixture of methanol/water (1/1, 0.8 mL) and stirred for 1 or 18 h. The reaction progress was followed by HPLC-MS. HMK **3** (MW = 189) showed a retention time of 4.90 min, while retention time for HMK **4** (MW = 233) was 5.05 min. On the other hand, the *S*-alkylated derivative (MW = 214) presented a retention time of 4.15 min. An additional experiment was carried out when the 2-mercaptoethanol concentration was increased 10 times. In this case, 100 μL of an acetonitrile solution of 2-mercaptoethanol (500 mM) was added. The same procedure described above was followed when triethylamine was used; 100 μL of an acetonitrile solution of triethylamine (50 mM) was added to a methanol/water (1/1, 0.7 mL) solution.

MALDI-TOF Analyses. Human recombinant GSK-3β was purchased from Millipore (Millipore Iberica S.A.U). A stock solution of 10 μg of the enzyme in MiliiQ water (100 μL) was prepared. Compound **38** (17.8 mM) and compound **40** (10 μM) were dissolved in MeOH, then 1 μL of each compound solution was added to 10 μL of the enzyme stock solution reproducing IC_{50} experimental conditions prior to MALDI-TOF analysis.

Biological Studies. *Inhibition of GSK-3 (Radiometric Assay).* GSK-3β enzyme (Sigma) was incubated with 15 μM of ATP, 0.2 μCi of [γ-^{32}P]ATP, GS-1 substrate, and different concentrations of test compound. GSK-3β activity was assayed in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, and 1 mM EDTA buffer at 37 °C, in the presence of 15 mM GS-1 (substrate), 15 μM of ATP, 0.2 μCi of [γ-^{32}P] ATP in a final volume of 12 μL. After 20 min of incubation at 37 °C, 4 μL aliquots of the supernatant were spotted onto 2 × 2 pieces of Whatman P81 phosphocellulose paper, and the filter was washed four times (at least 10 min each time) in 1% phosphoric acid. The dried filters were transferred into scintillation vials, and the radioactivity was measured in a liquid scintillation counter. Blank values were subtracted, and the GSK-3β activity was expressed in percentage of maximal activity. The IC_{50} is defined as the concentration of each compound that reduces enzyme activity by 50% with respect to that without inhibitor present.

Inhibition of GSK-3 (Luminescent Assay). Human recombinant GSK-3β was purchased from Millipore (Millipore Iberica S.A.U.) The prephosphorylated polypeptide substrate was purchased from Millipore (Millipore Iberica S.A.U.). Kinase-Glo Luminescent Kinase Assay was obtained from Promega (Promega Biotech Ibérica, SL). ATP and all other reagents were from Sigma-Aldrich (St. Louis, MO). Assay buffer contained 50 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA, and 15 mM magnesium acetate.

The method of Baki et al.[52] was followed for the inhibition of GSK-3β. Kinase-Glo assays were performed in assay buffer using black 96-well plates. In a typical assay, 10 μL (10 μM) of test compound (dissolved in dimethyl sulfoxide [DMSO] at 1 mM concentration and diluted in advance in assay buffer to the desired concentration) and 10 μL (20 ng) of enzyme were added to each well followed by 20 μL of assay buffer containing 25 μM substrate and 1 μM ATP. The final DMSO concentration in the reaction mixture did not exceed 1%. After a 30 min incubation at 30 °C, the enzymatic reaction was stopped with 40 μL of Kinase-Glo reagent. Glow-type luminescence was recorded after 10 min using a FLUOstar Optima (BMG Labtechnologies GmbH, Offenburg, Germany) multimode reader. The activity is proportional to the difference of the total and consumed ATP. The inhibitory activities were calculated on the basis of maximal activities measured in the absence of inhibitor. The IC_{50} was defined as the concentration of each compound that reduces a 50% the enzymatic activity with respect to that without inhibitors.

GSK-3 Reversibility Studies. To study the type of enzymatic inhibition for the compounds, assays were performed to determine the activity of the enzyme after several times of incubation of the enzyme with the inhibitor. A reversible inhibitor does not increase the inhibition of the enzyme with the time of incubation, while an irreversible inhibitor increases the inhibition percentage as it increases the time of incubation with the enzyme.

Antibodies and Western Blot Analysis. The antibodies used in this study were: anti-β-actin mAb (Sigma); PHF-1, which is an antiphospho Ser$^{396/404}$ Tau mAb and Tau monoclonal antibody Tau-1, from Chemicon, that recognizes residues Ser 195, 198, 199, 202 non-phosphorylated.

Cell extracts were prepared from cells washed with 1 × PBS and then resuspended in a buffer containing the following: 20 mM HEPES, pH 7.4; 100 mM NaCl; 100 mM NaF; 1 mM sodium *ortho*-vanadate; 5 mM EDTA; Okadaic Acid 1 μM; 1% Triton X-100; and a protease inhibitor cocktail (Complete, Roche). The soluble fraction was obtained by centrifugation at 14 000 *g* for 10 min at 4 °C, and the proteins (10−50 μg) were separated by SDS-PAGE before being transferred to a nitrocellulose filter. The filters were blocked with 5% nonfat milk in PBS and 0.1% Tween-20 (PBS-T) and then incubated with primary antibodies overnight at 4 °C. Subsequently, the filters were rinsed three times in PBST buffer before being exposed to the corresponding peroxidase-conjugated secondary antibody (diluted 1:5000, Promega) for 1 h at room temperature. Immunoreactivity was visualized using an enhanced chemiluminescence detection system (Perkin-Elmer Life Sci.). Each experiment was normalized with respect to the amount of actin present in each cell extract. The data are expressed as the mean of three independent experiments and the data from control cells were considered 100 relative unit (r.u.).

Inhibition of Protein Kinases. The experimental procedures for the inhibition of different protein kinases are described in each paragraph:

- Abl kinase:[53] Mouse recombinant (*E.coli*), staurosporine was used as reference compound and poly GT (0.4 μg.mL^{-1}) as a substrate. Fluorescence polarization was used as the method of detection for the reaction product (phosphopoly GT).
- CAM kinase II:[54] From rat brain, staurosporine was used as reference compound and [γ^{33}-P] ATP + autocamtide-2 (5 μM) as a substrate. Liquid scintillation was used as method of detection for the reaction product ([γ^{33}-P] autocamtide-2).
- EGFR kinase:[55] From A-431 cells, PD 153035 was used as reference compound and [γ^{33}-P] ATP + poly GAT (0.48 mg.mL^{-1}) as a substrate. Liquid scintillation was used as method of detection for the reaction product ([γ^{33}-P] poly GAT).

- IRK (*h*):[56] Human recombinant, staurosporine was used as reference compound and [γ^{33}-P] ATP + poly GT (0.03 mg.mL^{-1}) as a substrate. Liquid scintillation was used as method of detection for the reaction product ([γ^{33}-P] poly GT).
- MAP kinase (ERK 42):[57] Rat recombinant (*E.coli*), staurosporine was used as reference compound and [γ^{33}-P] ATP + MBP (0.5 mg. mL^{-1}) as a substrate. Liquid scintillation was used as method of detection for the reaction product ([γ^{33}-P] MBP).
- MEK1 kinase:[58] Rabbit recombinat (*E.coli*), staurosporine was used as reference compound and ATP + unactivated MAP kinase (0.01 mg.mL^{-1}) as a substrate. Liquid scintillation was used as method of detection for the reaction product ([γ^{33}-P] MBP).
- Protein kinase p56^{lck}:[59] From bovine thymus, staurosporine was used as reference compound and [γ^{33}-P] ATP + poliGT (0.5 mg. mL^{-1}) as a substrate. Liquid scintillation was used as method of detection for the reaction product ([γ^{33}-P] poliGT).

Neurotransmitter Receptors Binding Assays. The neurotransmitter binding assays are described in each paragraph:

- α-2 (non selective) adrenergic receptor:[60] From rat cerebral cortex, yohimbine was used as reference compound and [^{3}H] RX 821002 as a ligand (0.5 nM).
- D2 (*h*) dopamine receptor:[61] Human recombinant (CHO cells), (+)-buctaclamol was used as reference compound and [^{3}H] spiperone as a ligand (0.3 nM).
- D3 (*h*) dopamine receptor:[62] Human recombinant (CHO cells), (+)-buctaclamol was used as reference compound and [^{3}H] spiperone as a ligand (0.3 nM).
- AMPA glutaminergic receptor:[63] From rat cerebral cortex, L-glutamate was used as reference compound and [^{3}H] AMPA as a ligand (8 nM).
- NMDA glutaminergic receptor:[64] From rat cerebral cortex, CGS 19755 was used as reference compound and [^{3}H] CGP 39653 as a ligand (5 nM).
- M (nonselective) muscarinic receptor:[65] From rat cerebral cortex, atropine was used as reference compound and [^{3}H] QNB as a ligand (0.05 nM).
- N (neuronal) (α-BGTX-insensitive) nicotinic receptor:[66] From rat cerebral cortex, nicotine was used as reference compound and [^{3}H] cytisine as a ligand (1.5 nM).
- N (neuronal) (α-BGTX-sensitive) nicotinic receptor:[67] From rat cerebral cortex, α-bungarotoxin was used as reference compound and [^{125}I] α-bungarotoxin as a ligand (1 nM).
- 5-HT (nonselective) serotoninergic receptor:[68] From rat cerebral cortex, serotonine was used as reference compound and [^{3}H] serotonine as a ligand (2 nM).

Molecular Modeling. Docking was performed with the program *rDock*, which is an extension of the program *RiboDock*,[69] using an empirical scoring function calibrated on the basis of protein−ligand complexes.[70] The reliability of *rDock* was assessed by docking a set of known GSK-3 inhibitors taking advantage of the X-ray crystallographic structures of their complexes with the enzyme (PDB entries 1J1B, 3F88, 1R0E, 1Q5K, 1UV5, and 2O5K; see Table S1 in Supporting Information). The docking of the novel HMK-containing adenine, benzimidazole, and maleimide derivatives was made using the X-ray structure of the original compounds that were designed (1J1B, 3F88, and 1R0E). The docking volume was defined as the space within 10 Å of the ligands found in those GSK-3 complexes. The structure of the ligands was initially energy minimized at the AM1[71] level using *Gaussian 03*.[72] Each compound was subjected to 100 docking runs, and the output docking modes were analyzed by visual inspection in conjunction with the docking scores.

■ ASSOCIATED CONTENT

Supporting Information. Poses for selected GSK-3 inhibitors upon docking to their targets; docked poses for derivatives of adenine (**32**) and maleimide (**40**) containing the halomethyketone moiety; elemental analyses of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

GSK-3, glycogen synthase kinase 3; CNS, central nervous system; AD, Alzheimer's disease; HMK, halomethylketone; TDZD, thiadiazolidindione; CDK-*n*, cyclin dependent kinase *n*, where *n* is 1, 2 or 5; GS-1, glycogen synthase 1; PDB, protein data bank; PK, protein kinase

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