Synthesis and Biological Evaluation of 3-Benzisoxazolyl-4-indolylmaleimides as Potent, Selective Inhibitors of Glycogen Synthase Kinase-3β

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Abstract: A series of novel 3-benzisoxazolyl-4-indolyl-maleimides were synthesized and evaluated for their GSK-3β inhibitory activity. Most compounds exhibited high inhibitory potency towards GSK-3β. Among them, compound 7j with an IC_{50} value of 0.73 nM was the most promising GSK-3β inhibitor. Preliminary structure-activity relationships were examined and showed that different substituents on the indole ring and N1-position of the indole ring had varying degrees of influence on the GSK-3β inhibitory potency. Compounds 7c, 7f, 7j–l and 7o–q could obviously reduce Aβ-induced Tau hyperphosphorylation by inhibiting GSK-3β in a cell-based functional assay.

Keywords: 3-benzisoxazolyl-4-indolylmaleimides; synthesis; GSK-3β; biological activity; docking; SAR
1. Introduction

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase which was identified in the late 1970s. Mammalian GSK-3 exists as two isoforms, GSK-3α and GSK-3β which share high homology at the catalytic domain but significantly differ in their N-terminal domain (84% overall and 98% in catalytic domain). Both isomers ubiquitously exist in cells and tissues and have similar biochemical properties [1–4]. GSK-3β plays a critical role in glycogen metabolism, embryogenesis, mitotic regulation, inflammation and neuroplasticity [5–8]. Inhibition of GSK-3β may provide therapy approach for several diseases such as cancer, type 2 diabetes, chronic inflammatory processes, stroke, bipolar disorders and Alzheimer's disease [9–13]. Accordingly, searching for novel and selective GSK-3β inhibitors is a very active area in both academic centers and pharmaceutical companies.

Staurosporine, a microbial alkaloid, was identified as a potent but nonselective GSK-3β inhibitor. Various bisindolylmaleimides such as GF 109203X and Ro 31-8220 have also been developed as potent GSK-3β inhibitors based on staurosporine (Figure 1) [14–16]. However, most of these bisindolylmaleimides are not suitable for the treatment of diseases such as diabetes and Alzheimer’s disease due to their toxicity, poor solubility and low selectivity, especially against PKC family [17,18]. The replacement of one indole with other heteroaryl groups resulted in a series of monoindolylmaleimides such as 4-azaindolyl-indolyl-maleimides, benzofuranyl-indolyl-maleimides and imidazo[1,2-a]pyridinyl-indolyl-maleimides (Figure 1), which showed potent and selective GSK-3β inhibitory activities [19–21].

Among them, GSK-3β inhibitor 603281-31-8 developed by Eli Lilly & Co had reached preclinical studies for the treatment of diabetes and was proved efficacy in ZDF rats [19]. In view of these facts
and also as a part of our work on the development of potent and selective GSK-3β inhibitors, herein we report the synthesis and biological evaluation of a new series of 3-benzisoxazoly1-4-indolyl-maleimides as GSK-3β inhibitors. Their structure-activity relationship and in silico molecular modeling study are also discussed in this study.

2. Results and Discussion

2.1. Chemistry

The general synthetic approach to target compounds 7a–q is outlined in Scheme 1.

![Scheme 1. Synthetic route to compounds 7a–q.](image)

Reagents and conditions: (a) i (COCl)₂, Et₂O; ii CH₃ONa, CH₃OH; (b) (Boc)₂O, DMAP, THF; (c) NaH, DMF, R₂X; (d) NaH, Br(CH₂)₂Br, DMF; (e) K₂CO₃, morpholine, DMF; (f) i HCl, (COCl)₂, Et₂O; ii CH₃ONa, CH₃OH; (g) i t-BuOK, THF; ii concentrated HCl.
Indole derivatives 1a–f were reacted with oxalyl chloride in Et₂O, followed by sodium methoxide to give compounds 2a–f. Reaction of 2a with (Boc)₂O in the presence of a catalytic amount of DMAP in THF afforded 3a. N-alkylation of 2a–f with different alkyl halides resulted in key intermediates 3b–h and 3j–q. In addition, treatment of indole with 1,4-dibromobutane afforded 4. N-substitution reaction of 4 with morpholine using K₂CO₃ as acid-trapping agent resulted in 5, which was then treated with oxalyl chloride, followed by sodium methoxide to give another key intermediate 3i. Condensation of glyoxylic esters 3a–q with 2-(benzo[d]isoxazol-3-yl)acetamide 6 [22] in the presence of t-BuOK in THF afforded the target compounds 7a–q.

2.2. Biological Activity and Molecular Modeling

2.2.1. Enzymatic Activity

The GSK-3β inhibitory potency of all target compounds was examined. In addition, selected compounds 7c, 7j and 7o were also tested for their inhibitory potency against other kinases (PKC-epsilon, JAK2, BRAF, IKK2, Drak2) to assess kinase selectivity. Staurosporine, a well known kinase inhibitor was used as the reference compound [19]. The results are listed in Tables 1 and 2. As indicated in Table 1, most of the tested compounds showed similar or more potent GSK-3β inhibitory activity as compared to that of staurosporine. The potency of GSK-3β inhibition of tested compounds was mainly influenced by the substitutions on the indole ring and N₁-position of the indole ring.

As expected, the introduction of suitable hydrophilic side chains at N₁-position of the indole ring gave an obvious enhancement of GSK-3β inhibitory activity (i.e., 7a, 7h, 7j–l, 7q). Among them, compound 7j with an imidazole group at the end of N₁-propyl chain exhibited the most potent GSK-3β inhibitory activity, with an IC₅₀ of 0.73 nM, which was about 460-fold more potent than that of compound 7a (IC₅₀ = 332.2 nM). Replacement of the terminal imidazole group in 7j with other substituents such as 1,2,4-triazole (7l, IC₅₀ = 20.9 nM), hydroxyl (7q, IC₅₀ = 38.9 nM), piperidine (7m, IC₅₀ = 511.8 nM) and pyrrolidine (7o, IC₅₀ = 658.8 nM) resulted in a 29- to 915-fold less inhibitory potency.

The results of inhibitory activities of compounds 7g–i, 7j and 7k showed that the length of the N₁-alkyl linker affected GSK-3β inhibitory potency. For example, compound 7j (IC₅₀ = 0.73 nM) with a (CH₂)₃ linker showed better inhibitory activity than compound 7k (IC₅₀ = 89.8 nM) with a (CH₂)₄ linker. The same conclusion could also be drawn from comparison of the inhibitory potency of 7h and 7i.

Interestingly, the introduction of a hydrophobic methyl group on the N₁-position of the indole ring in 7a resulted in a 15-fold increase in inhibitory potency for GSK-3β, while the replacement of the methyl with a large butyl group showed a 3-fold decrease in potency for GSK-3β inhibition.

When comparing the inhibitory activity of 7b–7f with 7h, it suggested that different substituents on the indole ring affected the inhibitory potency for GSK-3β. Compound 7c (IC₅₀ = 10.2 nM) with bromine at 5-position of the indole ring showed a 14-fold increase in inhibitory activity toward GSK-3β as compared to that of 7h (IC₅₀ = 137.7 nM). Fluorine at 6-position of the indole ring did not influence activity of 7h, while bromine or chlorine at 6-position or methoxy at the 5-position showed less inhibitory potency.
Table 1. GSK-3β inhibitory activity of the target compounds.

![Chemical structure](image)

| Compounds | R \(^1\) | R \(^2\) | IC\(_{50}\) (nM) ± SE \(^a\) |
|-----------|---------|---------|-----------------|
| Staurosporine | | | 72.2 ± 3.6 |
| 7a | H | H | 332.2 ± 25.7 |
| 7b | 5-OMe | 3-morpholinopropyl | 357.2 ± 17.1 |
| 7c | 5-Br | 3-morpholinopropyl | 10.2 ± 5.9 |
| 7d | 6-Br | 3-morpholinopropyl | 209.6 ± 8.9 |
| 7e | 6-Cl | 3-morpholinopropyl | 510.0 ± 20.1 |
| 7f | 6-F | 3-morpholinopropyl | 126.7 ± 3.2 |
| 7g | H | 2-morpholinoethyl | 615.6 ± 6.1 |
| 7h | H | 3-morpholinopropyl | 137.7 ± 6.0 |
| 7i | H | 4-morpholinobutyl | 410.7 ± 8.2 |
| 7j | H | 3-(1H-imidazol-1-yl)propyl | 0.73 ± 0.02 |
| 7k | H | 4-(1H-imidazol-1-yl)butyl | 89.8 ± 4.5 |
| 7l | H | 3-(1H-1,2,4-triazol-1-yl)propyl | 20.9 ± 5.1 |
| 7m | H | 3-(piperidin-1-yl)propyl | 511.8 ± 15.6 |
| 7n | H | 3-(pyrrolidin-1-yl)propyl | 658.8 ± 15.7 |
| 7o | H | methyl | 22.1 ± 1.5 |
| 7p | H | butyl | 58.7 ± 2.8 |
| 7q | H | hydroxypropyl | 38.9 ± 2.6 |

\(^a\) SE: standard error mean.

Table 2. The selectivity to tested kinases of target compounds 7c, 7j and 7o.

| Kinases     | IC\(_{50}\) (nM) ± SE \(^a\) or inhibition% at 0.8 μg/mL (~2 μM) |
|-------------|-----------------------------------------------------------|
| Staurosporine | | | |
| GSK-3β      | 72.2 ± 3.6 | 10.2 ± 5.9 | 0.7 ± 0.02 | 22.1 ± 1.5 |
| PKC-epsilon | 0.88 ± 0.03 | 12.1% | 10.9% | 21.2% |
| JAK2        | 2.26 ± 0.14 | 0.1% | 4.9% | 9.4% |
| BRAF        | 14.37 ± 0.86 | 3.4% | 0.8% | 1.1% |
| IKK2        | 1.21 ± 0.13 | 13.1% | 3.5% | 2.9% |
| Drak2       | 25.82 ± 2.11 | 1.67% | 4.47% | 13.76% |

\(^a\) SE: standard error mean.

The data in Table 2 showed that staurosporine was a potent and nonselective kinase inhibitor as reported in the literature [14], which inhibits not only GSK-3β (IC\(_{50}\) = 72.2 nM) but also many other kinases (e.g., PKC-epsilon, JAK2, BRAF, IKK2 and Drak2). Selected compounds 7c, 7j and 7o with high potency for GSK-3β inhibition were also evaluated for kinase selectivity against PKC-epsilon, JAK2, BRAF, IKK2 and Drak2. The results indicated that they displayed high selectivity for GSK-3β over other tested kinases.
2.2.2. Cellular Activity

It has been implicated that GSK-3β is involved in multiple cellular processes, and its ability to hyperphosphorylate Tau protein and induce neurofibrillary tangle was intensively studied. Therefore, the cell-based assay examining Tau phosphorylation at Serine 396 represents a direct functional assay to measure the cellular activity of GSK-3β inhibitors [23]. Compounds 7c, 7f, 7j–l and 7o–q were tested for the ability to reduce Tau phosphorylation at Ser 396 in human neuroblastoma SH-SY5Y cells. LiCl, a well-known inhibitor of GSK-3β [24], was used as a positive control in this assay. As shown in Figure 2, all selected compound significantly reduced Aβ25-35-induced Tau hyper-phosphorylation, showing that these compounds can inhibit GSK-3β activity at the cellular level. The Aβ-induced Tau hyperphosphorylation results in neurofibrillary tangle formation, which plays an important role in Alzheimer’s disease. Our data suggests that these novel GSK-3β inhibitors may have potential actions on inhibition of neurofibrillary tangle formation and would be tested for the treatment of Alzheimer’s disease. In addition, the predictions about these compounds’ brain permeability were performed using ADME module with Discovery Studio 2.1 software package. According to the prediction results (data not shown), most of them exhibited moderate blood-brain barrier permeability and would be further investigated for the treatment of Alzheimer’s disease.

Figure 2. Effects of GSK-3β inhibitors on Tau phosphorylation (ser396) in SH-SY5Y cells.

2.2.3. Molecular Modeling

To examine possible binding modes of compounds bearing different side chains at N1-position of the indole ring (e.g., 7j and 7n) with GSK-3β, a docking analysis utilizing the C-DOCKER program within the Discovery Studio 2.1 software package was performed. The published X-ray crystal structure of GSK-3β (PDB ID: 1Q3D) [14] was used for the docking calculation. Figure 3 shows that both 7j and 7n could occupy the ATP binding site of GSK-3β with similar binding modes as a few other ATP-competitive inhibitors of GSK-3β [14,25], and they could thus serve as ATP-competitive inhibitors of GSK-3β. The NH and carbonyl group in maleimide ring of 7j and 7n could form two key hydrogen bonds with Asp133 and Val135 of GSK-3β. Besides, the 3-position nitrogen atom of the imidazole ring of 7j could form another hydrogen bond with Lys-183, which was not observed in 7n. Furthermore, the CDOCKERENERAGE of 7j (−20.821 kcal/mol) was much lower than that of 7n.
Thus, the molecular docking study results could explain the fact that 7j showed significantly improved potency compared to that of 7n.

Figure 3. Docking of 7j to GSK-3β crystal structure. (A) Ribbon show of compound 7j bound to GSK-3β; (B) Surface show of compound 7j dock into GSK-3β. Main text paragraph; (C) Ribbon show of compound 7n bound to GSK-3β; (D) Surface show of compound 7n dock into GSK-3β.

3. Experimental

3.1. General

Melting points were determined with a BÜCHI Melting Point B-450 apparatus (Büchi Labortechnik, Flawil, Switzerland). The $^1$H-NMR spectra were recorded in DMSO-$d_6$ or CDCl$_3$ on Bruker Avance DMX 500 at 500 MHz (chemical shifts are expressed as $\delta$ values relative to TMS as internal standard). ESI spectra (positive ion mode) were recorded on an Esquire-LC-00075 spectrometer. Elemental analyses were performed on an Eager 300 instrument. All reactions were monitored by thin-layer chromatography (TLC). All reagents were obtained from commercial sources and used without further purification unless stated. Et$_2$O and THF were distilled from sodium-benzophenone. DMF was distilled from calcium hydride.
3.2. General Procedure for the Preparation of 2a–f

Oxalyl chloride (3.40 g, 26 mmol) in Et₂O (5 mL) was added dropwise to a solution of indole adducts 1a–f (26 mmol) in Et₂O (30 mL) at 0–5 °C [26,27]. The reaction mixture was stirred under the same conditions for 1 h, and a 20 wt% solution of CH₃ONa in MeOH (14.05 g, 52 mmol) was added dropwise at −30 °C to −20 °C. After addition, the mixture was stirred for 30 min at room temperature, and poured into cold water (100 mL). The product was isolated with filtration, washed with dichloromethane and dried to afford 2a–f.

**Methyl 2-(1H-indol-3-yl)-2-oxoacetate (2a).** Light yellow solid, 86.3% yield, mp: 208–210 °C. 
\[\text{H-NMR (DMSO-}d_6\text{): } \delta 12.48 (\text{brs, 1H}), 8.46 (d, J = 3.5 Hz, 1H), 8.16 (d, J = 7.0 Hz, 1H), 7.55 (d, J = 7.0 Hz, 1H), 7.32–7.26 (m, 2H), 3.90 (s, 3H).\]

**Methyl 2-(5-methoxy-1H-indol-3-yl)-2-oxoacetate (2b).** Light yellow solid, 71.0% yield, mp: 221–223 °C.
\[\text{H-NMR (DMSO-}d_6\text{): } \delta 12.34 (\text{brs, 1H}), 8.37 (d, J = 3.0 Hz, 1H), 7.66 (d, J = 2.0 Hz, 1H), 7.45 (d, J = 9.0 Hz, 1H), 6.93 (dd, J = 9.0, 2.0 Hz, 1H), 3.89 (s, 3H), 3.81 (s, 3H).\]

**Methyl 2-(5-bromo-1H-indol-3-yl)-2-oxoacetate (2c).** Light yellow solid, 55.3% yield, mp: 215–217 °C.
\[\text{H-NMR (DMSO-}d_6\text{): } \delta 12.59 (\text{brs, 1H}), 8.52 (d, J = 3.0 Hz, 1H), 8.30 (d, J = 2.0 Hz, 1H), 7.54 (d, J = 8.5 Hz, 1H), 7.45 (dd, J = 8.5, 2.0 Hz, 1H), 3.90 (s, 3H).\]

**Methyl 2-(6-bromo-1H-indol-3-yl)-2-oxoacetate (2d).** Light yellow solid, 58.9% yield, mp: 207–209 °C.
\[\text{H-NMR (DMSO-}d_6\text{): } \delta 12.50 (\text{brs, 1H}), 8.50 (s, 1H), 8.10 (d, J = 8.5 Hz, 1H), 7.75 (d, J = 2.0 Hz, 1H), 7.43 (dd, J = 8.5, 2.0 Hz, 1H), 3.90 (s, 3H).\]

**Methyl 2-(6-chloro-1H-indol-3-yl)-2-oxoacetate (2e).** Light yellow solid, 62.8% yield, mp: 246–248 °C.
\[\text{H-NMR (DMSO-}d_6\text{): } \delta 12.52 (\text{brs, 1H}), 8.51 (d, J = 3.5 Hz, 1H), 8.15 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 2.0 Hz, 1H), 7.31 (dd, J = 8.5, 2.0 Hz, 1H), 3.90 (s, 3H).\]

**Methyl 2-(6-fluoro-1H-indol-3-yl)-2-oxoacetate (2f).** Light yellow solid, 60.3% yield, mp: 182–184 °C.
\[\text{H-NMR (DMSO-}d_6\text{): } \delta 12.48 (\text{brs, 1H}), 8.48 (s, 1H), 8.15 (dd, J = 8.5, 5.5 Hz, 1H), 7.36 (dd, J = 9.5, 2.0 Hz, 1H), 7.15 (td, J = 9.5, 2.0 Hz, 1H), 3.90 (s, 3H).\]

3.3. tert-Butyl 3-(2-methoxy-2-oxoacetyl)-1H-indole-1-carboxylate (3a)

A solution of 2a (2.0 g, 9.8 mmol), Boc anhydride (2.7 g, 12.3 mmol) and DMAP (0.01 g) in dry THF (100 mL) was reacted for 3 h at room temperature. The solvent was removed under vacuum, and the residue was recrystallized from petroleum ether/ethyl acetate to give 2.0 g (67.1%) 3a as a white solid, mp: 132–133 °C. 
\[\text{H-NMR (CDCl}_3\text{): } \delta 8.81 (s, 1H), 8.40 (dd, J = 6.5, 2.0 Hz, 1H), 8.17 (dd, J = 6.5, 1.5 Hz, 1H), 7.41–7.38 (m, 2H), 3.98 (s, 3H), 1.71 (s, 9H).\]

3.4. General Procedure for the Preparation of 3b–h, 3j–n and 3q

70% NaH (0.51 g, 14.8 mmol) was added portionwise to a solution of 2a–f (14.8 mmol) in DMF (30 mL) at 0–5 °C. The reaction mixture was warmed to room temperature and stirred for 30 min.
After that, the appropriate R²X [4-(3-chloropropyl)morpholine, 4-(2-chloroethyl)morpholine, 1-(3-chloropropyl)-1H-imidazole, 1-(4-chlorobutyl)-1H-imidazole, 1-(3-chloropropyl)-1H-1,2,4-triazole, 1-(3-chloropropyl)piperidine, 1-(3-chloropropyl)pyrrolidine or (3-bromopropoxy)(tert-butyl)dimethylsilane] (19.2 mmol) was added. Then the mixture was heated to 55–60 °C and reacted for 12 h. After cooling, the mixture was poured into cold water (300 mL) and extracted with ethyl acetate (3 × 100 mL). The organic phase was combined, washed with brine (3 × 300 mL), dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel using ethyl acetate/methanol (50:1, v/v) as eluent to afford 3b–h, 3j–n and 3q.

Methyl 2-(5-methoxy-1-(3-morpholinopropyl)-1H-indol-3-yl)-2-oxoacetate (3b). Light yellow solid, 64.7% yield, mp: 67–68 °C. ¹H-NMR (CDCl₃): δ 8.38 (s, 1H), 7.95 (d, J = 2.5 Hz, 1H), 7.32 (d, J = 9.0 Hz, 1H), 6.97 (dd, J = 9.0, 2.5 Hz, 1H), 4.28 (t, J = 7.0 Hz, 2H), 3.95 (s, 3H), 3.91 (s, 3H), 3.77–3.70 (m, 4H), 2.42–2.38 (m, 4H), 2.27 (t, J = 6.5 Hz, 2H), 2.06–2.01 (m, 2H). ESI-MS: m/z [M+H]+ 361. Anal. Calcd for C₁₉H₂₄N₂O₅: C, 63.32; H, 6.71; N, 7.77. Found: C, 63.49; H, 6.68; N, 7.49.

Methyl 2-(5-bromo-1-(3-morpholinopropyl)-1H-indol-3-yl)-2-oxoacetate (3c). Light yellow solid, 56.6% yield, mp: 112–113 °C. ¹H-NMR (CDCl₃) δ 8.43 (s, 1H), 8.31 (d, J = 8.5 Hz, 1H), 7.67 (d, J = 2.0 Hz, 1H), 7.45 (dd, J = 8.5, 2.0 Hz, 1H), 4.29 (t, J = 7.0 Hz, 2H), 3.95 (s, 3H), 3.81–3.75 (m, 4H), 2.43–2.39 (m, 4H), 2.25 (t, J = 6.5 Hz, 2H), 2.05–2.01 (m, 2H). ESI-MS: m/z [M+H]+ 409. Anal. Calcd for C₁₈H₂₁BrN₂O₄: C, 52.82; H, 5.17; N, 6.84. Found: C, 52.96; H, 5.23; N, 6.76.

Methyl 2-(6-chloro-1-(3-morpholinopropyl)-1H-indol-3-yl)-2-oxoacetate (3e). Light yellow solid, 59.1% yield, mp: 115–116 °C. ¹H-NMR (CDCl₃): δ 8.42 (s, 1H), 8.34 (d, J = 8.5 Hz, 1H), 7.49 (d, J = 1.5 Hz, 1H), 7.30 (dd, J = 8.5, 1.5 Hz, 1H), 4.28 (t, J = 6.5 Hz, 2H), 3.94 (s, 3H), 3.80–3.70 (m, 4H), 2.43–2.37 (m, 4H), 2.24 (t, J = 6.5 Hz, 2H), 2.04–2.00 (m, 2H). ESI-MS: m/z [M+H]+ 365. Anal. Calcd for C₁₈H₂₁ClN₂O₄: C, 59.26; H, 5.80; N, 7.68. Found: C, 59.44; H, 5.78; N, 7.45.

Methyl 2-(1-(2-morpholinoethyl)-1H-indol-3-yl)-2-oxoacetate (3g). Light yellow solid, 52.4% yield, mp: 110–112 °C. ¹H-NMR (CDCl₃): δ 8.51 (s, 1H), 8.49–8.44 (m, 1H), 7.43–7.39 (m, 1H), 7.38–7.35 (m, 2H), 4.29 (t, J = 6.5 Hz, 2H), 3.97 (s, 3H), 3.75–3.69 (m, 4H), 2.81 (t, J = 6.5 Hz, 2H), 2.54–2.47
Molecules 2013, 18 5507

(m, 4H). ESI-MS: m/z [M+H]+ 317. Anal. Caled for C17H20N2O4: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.71; H, 6.68; N, 8.68.

Methyl 2-((3-morpholinopropyl)-1H-indol-3-yl)-2-oxoacetate (3h). Light yellow solid, 67.5% yield, mp: 103–104 °C. 1H-NMR (CDCl3): δ 8.47–8.43 (m, 2H), 7.46–7.41 (m, 1H), 7.38–7.32 (m, 2H), 4.32 (t, J = 6.5 Hz, 2H), 3.95 (s, 3H), 3.78–3.72 (m, 4H), 2.44–2.38 (m, 4H), 2.28 (t, J = 6.5 Hz, 2H), 2.08–2.02 (m, 2H). ESI-MS: m/z [M+H]+ 317. Anal. Caled for C17H20N2O4: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.71; H, 6.68; N, 8.68.

Methyl 2-((3-(1H-imidazol-1-yl)propyl)-1H-indol-3-yl)-2-oxoacetate (3j). Light yellow solid, 52.8% yield, mp: 103–104 °C. 1H-NMR (CDCl3): δ 8.47–8.43 (m, 2H), 7.46–7.41 (m, 1H), 7.38–7.32 (m, 2H), 4.32 (t, J = 6.5 Hz, 2H), 3.95 (s, 3H), 3.78–3.72 (m, 4H), 2.44–2.38 (m, 4H), 2.28 (t, J = 6.5 Hz, 2H), 2.08–2.02 (m, 2H). ESI-MS: m/z [M+H]+ 317. Anal. Caled for C17H20N2O4: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.71; H, 6.68; N, 8.68.

Methyl 2-((3-(1H-imidazol-1-yl)propyl)-1H-indol-3-yl)-2-oxoacetate (3k). Light yellow solid, 41.6% yield, mp: 72–73 °C. 1H-NMR (CDCl3): δ 8.47–8.43 (m, 2H), 7.46–7.41 (m, 1H), 7.38–7.32 (m, 2H), 4.32 (t, J = 6.5 Hz, 2H), 3.95 (s, 3H), 3.78–3.72 (m, 4H), 2.44–2.38 (m, 4H), 2.28 (t, J = 6.5 Hz, 2H), 2.08–2.02 (m, 2H). ESI-MS: m/z [M+H]+ 317. Anal. Caled for C17H20N2O4: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.71; H, 6.68; N, 8.68.

Methyl 2-((3-(1H-1,2,4-triazol-1-yl)propyl)-1H-indol-3-yl)-2-oxoacetate (3l). Light yellow solid, 64.5% yield, mp: 98–99 °C. 1H-NMR (CDCl3): δ 8.47–8.43 (m, 2H), 7.46–7.41 (m, 1H), 7.38–7.32 (m, 2H), 4.32 (t, J = 6.5 Hz, 2H), 3.95 (s, 3H), 3.78–3.72 (m, 4H), 2.44–2.38 (m, 4H), 2.28 (t, J = 6.5 Hz, 2H), 2.08–2.02 (m, 2H). ESI-MS: m/z [M+H]+ 317. Anal. Caled for C17H20N2O4: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.71; H, 6.68; N, 8.68.

Methyl 2-oxo-2-((3-(piperidin-1-yl)propyl)-1H-indol-3-yl)acetate (3m). Light yellow solid, 52.4% yield, mp: 66–67 °C. 1H-NMR (DMSO-d6): δ 8.47 (s, 1H), 8.20 (d, J = 7.5 Hz, 1H), 7.68 (d, J = 7.5 Hz, 1H), 7.39–7.27 (m, 2H), 4.34 (t, J = 7.0 Hz, 2H), 3.90 (s, 3H), 2.29–2.18 (m, 4H), 2.13 (t, J = 6.5 Hz, 2H), 1.96–1.90 (m, 2H), 1.55–1.41 (m, 4H), 1.40–1.30 (m, 2H). ESI-MS: m/z [M+H]+ 311. Anal. Caled for C19H24N2O3: C, 69.49; H, 7.37; N, 8.53. Found: C, 69.69; H, 7.68; N, 8.48.
3.5. 1-(4-Bromobutyl)-1H-indole (4)

70% NaH (1.77 g, 51.6 mmol) was added portionwise to a solution of indole (5.0 g, 43.0 mmol) in DMF (50 mL) at 0–5 °C. The mixture was warmed to room temperature and stirred for 30 min. After that, it was added dropwise to a mixture of 1,4-dibromobutane (46.4 g, 215 mmol) in DMF (10 mL) at room temperature, and stirred for 12 h. The mixture was then poured into cold water (300 mL) and extracted with ethyl acetate (3 × 100 mL). The organic phase was combined, washed with brine (3 × 300 mL), dried over Na2SO4 and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate (80:1, v/v) as eluent to afford 7.6 g (71.2%) of 4 as a colorless liquid. 1H-NMR (CDCl3): δ 7.66 (d, J = 8.0 Hz, 1H), 7.36 (d, J = 8.0 Hz, 1H), 7.25–7.22 (m, 1H), 7.14–7.11 (m, 2H), 6.53 (d, J = 3.0 Hz, 1H), 4.19 (t, J = 7.0 Hz, 2H), 3.40 (t, J = 6.5 Hz, 2H), 2.07–2.00 (m, 2H), 1.90–1.85 (m, 2H).

3.6. 4-(4-(1H-Indol-1-yl)butyl)morpholine (5)

A mixture of 4 (2.0 g, 7.9 mmol), morpholine (6.9 g, 79.0 mmol) and potassium carbonate (1.9 g, 13.8 mmol) in DMF (30 mL) was stirred at 50 °C for 6 h. After cooling, the mixture was poured into cold water (200 mL) and extracted with ethyl acetate (3 × 100 mL). The organic phase was combined, washed with brine (3 × 300 mL), dried over Na2SO4 and concentrated in vacuum. The residue was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate/triethylamine (20:100:1, v/v/v) as eluent to afford 1.63 g (80.1%) of 5 as a colorless liquid. 1H-NMR (CDCl3): δ 7.66 (d, J = 8.0 Hz, 1H), 7.37 (d, J = 8.0 Hz, 1H), 7.24–7.21 (m, 1H), 7.14–7.11 (m, 2H), 6.51 (d, J = 3.0 Hz, 1H), 4.17 (t, J = 7.0 Hz, 2H), 3.75–3.70 (m, 4H), 2.39–2.33 (m, 6H), 1.93–1.87 (m, 2H), 1.57–1.50 (m, 2H). ESI-MS: m/z [M+H]+ 259. Anal. Calcd for C16H22N2O: C, 74.38; H, 8.58; N, 10.84. Found: C, 74.53; H, 8.64; N, 10.93.

3.7. Methyl 2-(1-(4-morpholinobutyl)-1H-indol-3-yl)-2-oxoacetate (3i)

1M HCl in dioxane (5.5 mL, 5.5 mmol) was added to a solution of 5 (1.29 g, 5.0 mmol) in CH3CN (30 mL) and Et2O (30 mL) at room temperature and stirred for 30 min. After that, oxalyl chloride (0.76 g, 6.0 mmol) in Et2O (5 mL) was added dropwise at 0–5 °C and the reaction mixture was stirred for 2 h at the same temperature. MeOH (10 mL) was added dropwise to the mixture at 0–5 °C and the result solution was stirred for 2 h at room temperature, then poured into a cold aqueous NaHCO3 solution and extracted with ethyl acetate (3 × 50 mL). The organic phase was combined, washed with brine (150 mL), dried over Na2SO4 and concentrated under vacuum. The residue was purified by flash column chromatography on silica gel using ethyl acetate/methanol (50:1, v/v) as eluent to afford 0.94 g (54.6%) of 3i as a light yellow solid, mp: 84–85 °C. 1H-NMR (CDCl3): δ 8.48–8.44 (m, 1H), 8.40 (s, 1H), 7.44–7.38 (m, 1H), 7.36–7.34 (m, 2H), 4.23 (t, J = 7.5 Hz, 2H), 3.97 (s, 3H), 3.73–3.65 (m, 4H), 2.39–2.34 (m, 6H), 2.02–1.92 (m, 2H), 1.59–1.51 (m, 2H). ESI-MS: m/z [M+H]+ 345. Anal. Calcd for C19H24N2O4: C, 66.26; H, 7.02; N, 8.13. Found: C, 66.40; H, 7.11; N, 8.32.
3.8. General Procedure for the Preparation of 3o and 3p

70% NaH (0.17 g, 4.9 mmol) was added portionwise to a solution of 2a (1.0 g, 4.9 mmol) in DMF (10 mL) at 0~5 °C. The reaction mixture was warmed to room temperature and stirred for 30 min. After that, iodomethane or 1-bromobutane (5.9 mmol) was added at 0 °C and stirred for 1 h at room temperature. The mixture was then poured into cold water (100 mL) and extracted with ethyl acetate (3 × 50 mL). The organic phase was combined, washed with brine (3 × 150 mL), dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate (3:1, v/v) as eluent to afford 3o and 3p.

**Methyl 2-(1-methyl-1H-indol-3-yl)-2-oxoacetate (3o).** Light yellow solid, 76.8% yield, mp: 73–74 °C. ¹H-NMR (CDCl₃) δ 8.51–8.42 (m, 1H), 8.35 (s, 1H), 7.39–7.36 (m, 3H), 3.96 (s, 3H), 3.88 (s, 3H). ESI-MS: m/z [M+H]⁺ 218. Anal. Calcd for C₁₂H₁₁NO₃: C, 66.35; H, 5.10; N, 6.45. Found: C, 66.55; H, 5.20; N, 6.59.

**Methyl 2-(1-butyl-1H-indol-3-yl)-2-oxoacetate (3p).** White solid, 80.5% yield, mp: 81–82 °C. ¹H-NMR (CDCl₃) δ 8.48–8.44 (m, 1H), 8.39 (s, 1H), 7.45–7.38 (m, 1H), 7.37–7.32 (m, 2H), 4.20 (t, J = 7.5 Hz, 2H), 3.96 (s, 3H), 1.86–1.82 (m, 2H), 1.42–1.38 (m, 2H), 0.98 (t, J = 7.5 Hz, 3H). ESI-MS: m/z [M+H]⁺ 260. Anal. Calcd for C₁₅H₁₇NO₃: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.62; H, 6.52; N, 5.65.

3.9. General Procedure for the Preparation of 7a–q

A solution of t-BuOK (94.0 mg, 0.84 mmol) in THF (5mL) was added dropwise to a solution of 3a–q (0.36 mmol) and 2-(benzo[d]isoxazol-3-yl)acetamide 6 (0.28 mmol) in dry THF (10 mL) at −10~0 °C. After stirring for 2 h at room temperature, concentrated hydrochloric acid (5 mL) was added and the result mixture was stirred for 30 min at room temperature, then poured into 10% NaHCO₃ aqueous solution (100 mL) and extracted with ethyl acetate (3 × 50 mL). The organic phase was combined and washed with brine (3 × 150 mL), dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel using dichloromethane/methanol (50:1, v/v) as eluent to afford 7a–q.

**3-(Benzo[d]isoxazol-3-yl)-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (7a).** Red solid, 44.4% yield, mp: 227–228 °C. ¹H-NMR (DMSO-d₆) δ 12.14 (brs, 1H), 11.44 (brs, 1H), 8.23 (s, 1H), 7.83 (d, J = 8.5 Hz, 1H), 7.69–7.58 (m, 2H), 7.44 (d, J = 8.0 Hz, 1H), 7.31 (t, J = 7.5 Hz, 1H), 7.08–7.04 (m, 1H), 6.72 (t, J = 8.0 Hz, 1H), 6.51 (d, J = 8.5 Hz, 1H). ESI-MS: m/z [M+H]⁺ 330. Anal. Calcd for C₁₉H₁₁N₃O₃: C, 69.30; H, 3.37; N, 12.76. Found: C, 69.06; H, 3.45; N, 12.64.

**3-(Benzo[d]isoxazol-3-yl)-4-(5-methoxy-1-(3-morpholinopropyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7b).** Saffron yellow solid, 11.6% yield, mp: 191–193 °C. ¹H-NMR (CDCl₃) δ 8.27 (s, 1H), 7.97 (brs, 1H), 7.66–7.62 (m, 2H), 7.57 (td, J = 8.0, 1.5 Hz, 1H), 7.25–7.23 (m, 2H), 6.76 (dd, J = 9.0, 2.5 Hz, 1H), 5.94 (d, J = 2.5 Hz, 1H), 4.29 (t, J = 6.5 Hz, 2H), 3.77 (t, J = 4.5 Hz, 4H), 3.11 (s, 3H), 2.48–2.42 (m, 4H), 2.32 (t, J = 6.5 Hz, 2H), 2.06–2.01(m, 2H). ESI-MS: m/z [M+H]⁺ 487. Anal. Calcd for C₂₇H₂₆N₄O₅: C, 66.65; H, 5.39; N, 11.52. Found: C, 66.76; H, 5.47; N, 11.62.
3-(Benzo[d]isoxazol-3-yl)-4-(5-bromo-1-(3-morpholinopropyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7c). Saffron yellow solid, 21.3% yield, mp: 180–182 °C. $^1$H-NMR (CDCl$_3$) δ 8.32 (s, 1H), 7.97 (brs, 1H), 7.68 (t, $J = 8.5$ Hz, 2H), 7.61 (td, $J = 8.5$, 1.5 Hz, 1H), 7.32 (t, $J = 8.0$ Hz, 1H), 7.26–7.25 (m, 2H), 6.81 (s, 1H), 4.29 (t, $J = 7.0$ Hz, 2H), 3.75 (t, $J = 4.5$ Hz, 4H), 2.42–2.39 (m, 4H), 2.30 (t, $J = 6.6$ Hz, 2H), 2.03–2.01 (m, 2H). ESI-MS: m/z [M+H]$^+$ 535. Anal. Calcd for C$_{26}$H$_{23}$N$_4$O$_4$Br: C, 58.33; H, 4.33; N, 10.46. Found: C, 58.21; H, 4.49; N, 10.53.

3-(Benzo[d]isoxazol-3-yl)-4-(6-bromo-1-(3-morpholinopropyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7d). Saffron yellow solid, 12.7% yield, mp: 226–228 °C. $^1$H-NMR (CDCl$_3$) δ 8.27 (s, 1H), 7.97 (brs, 1H), 7.68–7.64 (m, 2H), 7.62 (d, $J = 2.0$ Hz, 1H), 7.59 (td, $J = 1.5$ Hz, 1H), 7.29 (t, $J = 6.5$ Hz, 2H), 3.79 (t, $J = 4.5$ Hz, 4H), 2.47–2.40 (m, 4H), 2.29 (t, $J = 6.5$ Hz, 2H), 2.05–2.02 (m, 2H). ESI-MS: m/z [M+H]$^+$ 535. Anal. Calcd for C$_{26}$H$_{23}$N$_4$O$_4$Br: C, 58.33; H, 4.33; N, 10.46. Found: C, 58.39; H, 4.51; N, 10.42.

3-(Benzo[d]isoxazol-3-yl)-4-(6-chloro-1-(3-morpholinopropyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7e). Red solid, 11.5% yield, mp: 216–218 °C. $^1$H-NMR (CDCl$_3$) δ 8.29 (s, 1H), 7.97 (brs, 1H), 7.65 (t, $J = 7.5$ Hz, 2H), 7.62 (d, $J = 8.0$ Hz, 1H), 7.45 (d, $J = 1.5$ Hz, 1H), 7.28 (t, $J = 7.5$ Hz, 1H), 6.85 (dd, $J = 8.5$, 1.5 Hz, 1H), 6.64 (d, $J = 8.5$ Hz, 1H), 4.29 (t, $J = 6.5$ Hz, 2H), 3.78 (t, $J = 4.5$ Hz, 4H), 2.45–2.41 (m, 4H), 2.29 (t, $J = 6.5$ Hz, 2H), 2.06–2.02 (m, 2H). ESI-MS: m/z [M+H]$^+$ 491. Anal. Calcd for C$_{26}$H$_{23}$N$_4$O$_4$Cl: C, 63.61; H, 4.72; N, 11.41. Found: C, 63.52; H, 4.64; N, 11.51.

3-(Benzo[d]isoxazol-3-yl)-4-(6-fluoro-1-(3-morpholinopropyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7f). Yellow solid, 29.9% yield, mp: 219–221 °C. $^1$H-NMR (CDCl$_3$) δ 8.29 (s, 1H), 8.06 (brs, 1H), 7.66–7.66 (m, 2H), 7.58 (t, $J = 7.5$ Hz, 1H), 7.29 (t, $J = 7.5$ Hz, 1H), 7.11 (d, $J = 9.5$ Hz, 1H), 6.68–6.62 (m, 2H), 4.27 (t, $J = 6.5$ Hz, 2H), 3.77 (t, $J = 4.5$ Hz, 4H), 2.46–2.41 (m, 4H), 2.31 (t, $J = 6.5$ Hz, 2H), 2.06–2.03 (m, 2H). ESI-MS: m/z [M+H]$^+$ 475. Anal. Calcd for C$_{26}$H$_{23}$N$_4$O$_4$F: C, 65.81; H, 4.89; N, 11.81. Found: C, 65.99; H, 4.75; N, 11.87.

3-(Benzo[d]isoxazol-3-yl)-4-(1-(2-morpholinoethyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7g). Yellow solid, 24.0% yield, mp: 205–207 °C. $^1$H-NMR (CDCl$_3$) δ 8.39 (s, 1H), 7.79 (brs, 1H), 7.65 (t, $J = 7.5$ Hz, 2H), 7.58 (t, $J = 7.5$ Hz, 1H), 7.29 (t, $J = 7.5$ Hz, 1H), 7.11 (d, $J = 9.5$ Hz, 1H), 6.73 (d, $J = 8.0$ Hz, 1H), 4.31 (t, $J = 6.5$ Hz, 2H), 3.73 (t, $J = 7.5$ Hz, 4H), 2.82 (t, $J = 6.5$ Hz, 2H), 2.56–2.42 (m, 4H). ESI-MS: m/z [M+H]$^+$ 443. Anal. Calcd for C$_{25}$H$_{22}$N$_4$O$_4$: C, 67.86; H, 5.01; N, 12.66. Found: C, 67.96; H, 5.11; N, 12.85.

3-(Benzo[d]isoxazol-3-yl)-4-(1-(3-morpholinopropyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7h). Red solid, 38.1% yield, mp: 197–198 °C. $^1$H-NMR (CDCl$_3$) δ 8.89 (brs, 1H), 8.33 (s, 1H), 8.06 (brs, 1H), 7.79 (brs, 1H), 7.57 (t, $J = 7.5$ Hz, 2H), 7.35 (d, $J = 8.0$ Hz, 1H), 7.25 (t, $J = 7.5$ Hz, 1H), 7.19 (t, $J = 7.5$ Hz, 1H), 6.90 (t, $J = 7.5$ Hz, 1H), 6.73 (d, $J = 8.0$ Hz, 1H), 4.31 (t, $J = 6.5$ Hz, 2H), 3.73 (t, $J = 7.5$ Hz, 4H), 2.82 (t, $J = 6.5$ Hz, 2H), 2.56–2.42 (m, 4H). ESI-MS: m/z [M+H]$^+$ 457. Anal. Calcd for C$_{26}$H$_{24}$N$_4$O$_4$: C, 68.41; H, 5.30; N, 12.27. Found: C, 68.69; H, 5.25; N, 12.41.
3-(Benzo[d]isoxazol-3-yl)-4-(1-(4-morpholinobutyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7i). Red solid, 22.6% yield, mp: 89–91 °C. 1H-NMR (CDCl3) δ 8.28 (s, 1H), 8.25 (brs, 1H), 7.66–7.62 (m, 2H), 7.56 (t, J = 7.5 Hz, 1H), 7.35 (d, J = 8.0 Hz, 1H), 7.24 (t, J = 8.0 Hz, 1H), 7.18 (t, J = 7.0 Hz, 1H), 6.88 (t, J = 7.5 Hz, 1H), 6.72 (d, J = 8.0 Hz, 1H), 4.23 (t, J = 6.5 Hz, 2H), 3.71 (t, J = 4.5 Hz, 4H), 2.45–2.35 (m, 6H), 2.01–1.90 (m, 2H), 1.62–1.55 (m, 2H). ESI-MS: m/z [M+H]+ 471. Anal. C27H26N4O4: C, 68.92; H, 5.57; N, 11.91. Found: C, 68.75; H, 5.62; N, 11.82.

3-(1-(3-(1H-imidazol-1-yl)propyl)-1H-indol-3-yl)-4-(benzo[d]isoxazol-3-yl)-1H-pyrrole-2,5-dione (7j). Red solid, 16.1% yield, mp: 208–210 °C. 1H-NMR (CDCl3) δ 8.32 (brs, 1H), 8.24 (s, 1H), 7.67 (d, J = 8.5 Hz, 1H), 7.85 (d, J = 8.5 Hz, 1H), 7.61–7.53 (m, 2H), 7.29 (t, J = 7.0 Hz, 1H), 7.24–7.18 (m, 2H), 7.17 (s, 1H), 6.98 (s, 1H), 6.94 (td, J = 7.0, 1.0 Hz, 1H), 6.81 (d, J = 8.0 Hz, 1H), 4.20 (t, J = 6.5 Hz, 2H), 3.99 (t, J = 6.5 Hz, 2H), 2.47–2.40 (m, 2H). ESI-MS: m/z [M+H]+ 438. Anal. C25H19N5O3: C, 68.64; H, 4.38; N, 16.01. Found: C, 68.81; H, 4.35; N, 16.26.

3-(1-(4-(1H-imidazol-1-yl)butyl)-1H-indol-3-yl)-4-(benzo[d]isoxazol-3-yl)-1H-pyrrole-2,5-dione (7k). Red solid, 15.6% yield, mp: 203–205 °C. 1H-NMR (CDCl3) δ 8.35 (brs, 1H), 8.24 (s, 1H), 7.69–7.63 (m, 2H), 7.57 (td, J = 7.0, 1.5Hz, 1H), 7.47 (s, 1H), 7.29 (d, J = 6.0 Hz, 1H), 7.25 (d, J = 7.5 Hz, 1H), 7.19 (t, J = 7.5 Hz, 1H), 7.08 (s, 1H), 6.92 (t, J = 7.5 Hz, 1H), 6.87 (s, 1H), 6.78(d, J = 8.0 Hz, 1H), 4.21 (t, J = 6.5 Hz, 2H), 3.92 (t, J = 6.5 Hz, 2H), 1.95–1.87 (m, 2H), 1.86–1.80 (m, 2H). ESI-MS: m/z [M+H]+ 452. Anal. C26H21N5O3: C, 69.17; H, 4.69; N, 15.51. Found: C, 68.96; H, 4.65; N, 15.73.

3-(1-(3-(1H-1,2,4-Triazol-1-yl)propyl)-1H-indol-3-yl)-4-(benzo[d]isoxazol-3-yl)-1H-pyrrole-2,5-dione (7l). Red solid, 21.3% yield, mp: 203–210 °C. 1H-NMR (DMSO-d6) δ 11.46 (brs, 1H), 8.54 (s, 1H), 8.28 (s, 1H), 8.01 (s, 1H), 7.81 (d, J = 8.5 Hz, 1H), 7.66–7.61 (m, 2H), 7.52 (d, J = 8.0 Hz, 1H), 7.29 (t, J = 7.5 Hz, 1H), 7.13 (t, J = 7.5 Hz, 1H), 6.79 (t, J = 8.0 Hz, 1H), 6.57 (d, J = 8.0 Hz, 1H), 4.36 (t, J = 6.5 Hz, 2H), 4.20 (t, J = 6.5 Hz, 2H), 2.36–2.28 (m, 2H). ESI-MS: m/z [M+H]+ 439. Anal. C24H18N6O3: C, 65.75; H, 4.14; N, 19.17. Found: C, 65.58; H, 4.12; N, 19.34.

3-(Benzo[d]isoxazol-3-yl)-4-(1-(3-(piperidin-1-yl)propyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7m). Red solid, 21.3% yield, mp: 214–216 °C. 1H-NMR (CDCl3) δ 8.32 (brs, 1H), 8.24 (s, 1H), 8.28 (s, 1H), 8.01 (s, 1H), 7.81 (d, J = 8.5 Hz, 1H), 7.66–7.61 (m, 2H), 7.52 (d, J = 8.0 Hz, 1H), 7.29 (t, J = 7.5 Hz, 1H), 7.13 (t, J = 7.5 Hz, 1H), 6.79 (t, J = 8.0 Hz, 1H), 6.57 (d, J = 8.0 Hz, 1H), 4.36 (t, J = 6.5 Hz, 2H), 4.20 (t, J = 6.5 Hz, 2H), 2.36–2.28 (m, 2H). ESI-MS: m/z [M+H]+ 455. Anal. C27H26N4O3: C, 71.35; H, 5.77; N, 12.33. Found: C, 71.13; H, 5.63; N, 12.46.

3-(Benzo[d]isoxazol-3-yl)-4-(1-(3-(pyrrolidin-1-yl)propyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7n). Saffron yellow solid, 20.8% yield, mp: 171–173 °C. 1H-NMR (CDCl3) δ 8.41 (s, 1H), 7.63 (d, J = 8.5, 1H), 7.59 (d, J = 8.0 Hz, 1H), 7.55 (td, J = 8.0, 1.0 Hz, 1H), 7.38 (d, J = 8.5 Hz, 1H), 7.23 (t, J = 8.0 Hz, 1H), 7.16 (t, J = 8.0 Hz, 1H), 6.85 (t, J = 8.0 Hz, 1H), 6.67 (d, J = 8.0 Hz, 1H), 4.31 (t, J = 7.0 Hz, 2H), 2.55–2.35 (m, 6H), 2.17–2.10 (m, 2H), 1.73–1.61 (m, 4H), 1.52–1.47 (m, 2H). ESI-MS: m/z [M+H]+ 441. Anal. C26H24N4O3: C, 70.89; H, 5.49; N, 12.72. Found: C, 70.68; H, 5.34; N, 12.97.
3-(Benzo[d]isoxazol-3-yl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7o). Red solid, 27.8% yield, mp: 221–222 °C. \( ^1 \)H-NMR (CDCl\(_3\)) \( \delta \) 8.24 (s, 1H), 7.81 (brs, 1H), 7.65 (d, \( J = 9.5 \) Hz, 2H), 7.57 (t, \( J = 8.0 \) Hz, 1H), 7.32 (d, \( J = 8.5 \) Hz, 1H), 7.19 (t, \( J = 8.0 \) Hz, 1H), 6.97 (t, \( J = 8.0 \) Hz, 1H), 6.88 (t, \( J = 7.5 \) Hz, 1H), 6.65 (d, \( J = 8.5 \) Hz, 1H), 3.90 (s, 3H). ESI-MS: \( m/z \) [M+H]\(^+\) 344. Anal. C\(_{20}\)H\(_{13}\)N\(_3\)O\(_3\): C, 69.96; H, 3.82; N, 12.24. Found: C, 70.11; H, 3.77; N, 12.05.

3-(Benzo[d]isoxazol-3-yl)-4-(1-butyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7p). Saffron yellow solid, 35.2% yield, mp: 180–182 °C. \( ^1 \)H-NMR (CDCl\(_3\)) \( \delta \) 8.25 (s, 1H), 7.82 (brs, 1H), 7.65 (d, \( J = 8.5 \) Hz, 1H), 7.61 (d, \( J = 8.5 \) Hz, 1H), 7.56 (t, \( J = 8.0 \) Hz, 1H), 7.34 (d, \( J = 8.0 \) Hz, 1H), 7.24 (t, \( J = 7.5 \) Hz, 1H), 7.18 (t, \( J = 7.5 \) Hz, 1H), 6.89 (t, \( J = 7.5 \) Hz, 1H), 6.75 (d, \( J = 7.5 \) Hz, 1H), 4.22 (t, \( J = 7.5 \) Hz, 2H), 1.95–1.83 (m, 2H), 1.42–1.37 (m, 2H), 0.98 (t, \( J = 7.5 \) Hz, 3H). ESI-MS: \( m/z \) [M+H]\(^+\) 386. Anal. C\(_{23}\)H\(_{19}\)N\(_3\)O\(_3\): C, 71.67; H, 4.97; N, 10.90. Found: C, 71.55; H, 4.84; N, 11.03.

3-(Benzo[d]isoxazol-3-yl)-4-(1-(3-hydroxypropyl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione (7q). Yellow solid, 10.0% yield, mp: 219–220 °C. \( ^1 \)H-NMR (CDCl\(_3\)) \( \delta \) 8.29 (s, 1H), 7.64 (t, \( J = 8.0 \) Hz, 2H), 7.57 (t, \( J = 7.5 \) Hz, 1H), 7.40 (t, \( J = 8.5 \) Hz, 1H), 7.24 (t, \( J = 8.0 \) Hz, 1H), 7.19 (t, \( J = 8.0 \) Hz, 1H), 6.91 (t, \( J = 7.5 \) Hz, 1H), 6.79 (d, \( J = 7.5 \) Hz, 1H), 4.39 (t, \( J = 7.0 \) Hz, 2H), 3.67 (t, \( J = 7.0 \) Hz, 2H), 2.17–2.10 (m, 2H). ESI-MS: \( m/z \) [M+H]\(^+\) 388. Anal. C\(_{22}\)H\(_{17}\)N\(_3\)O\(_4\): C, 68.21; H, 4.42; N, 10.85. Found: C, 68.07; H, 4.51; N, 11.09.

3.10. Biological Activity Assay

3.10.1. Kinase Assay

Human kinome, consisting of 518 kinases, is classified into CMGC, AGC, TK, TKL, CAMK, STE and other 7 subfamilies, according to the DNA sequence and evolution. GSK3\( \beta \) belongs to CMGC family. Here, the other family members were used to evaluate the selectivity of GSK3\( \beta \) inhibitors, including PKC-epsilon (AGC family), JAK2 (TK family), Braf (TKL family), DRAK2 (CAMK family) and IKK\( \beta \) (other family). The recombinant GST-GSK-3\( \beta \) protein was expressed in Escherichia coli strain BL21-Codon Plus (DE3), purified by GSTrap affinity chromatography, and cleaved by thrombin. The GSK-3\( \beta \) kinase assay was carried out with the Z'-LYTETM Kinase Assay kit Ser/Thr 9 Peptide substrate (Invitrogen, Grand, NY, USA) in 10 \( \mu \)L reaction volume containing 50 nM enzyme, 30 \( \mu \)M ATP and 2 \( \mu \)M substrate peptide. Drak2 Proteins were presented by professor Jiang-ping Wu (Laboratory of Immunology, Research Centre, CHUM, Notre Dame Hospital, Pavilion DeSève). The Drak2 kinase reaction was performed in a final assay volume of 3.4 \( \mu \)L using the ADP-GLO Kinase Assay Kit (Promega, Madison, WI, USA), according the ADP-GLO protocol and read on an EnVision plate reader. The recombinant PKC-epsilon, IKK\( \beta \), and JAK2 with N-terminal His-tag were expressed using baculovirus expression system and purified with Ni-Beads. BRAF protein was from Carna Biosciences, Inc. (Kobe Port Island, Japan). And the related kinase reactions were performed in a final assay volume of 10 \( \mu \)L using the related HTRF Assay Kit (Cisbio, Codolet, France). Reactions were according the HTRF protocol and read on an EnVision plate reader. All reactions were carried out in triplicate. IC\(_{50}\) values (concentration at which a 50% of enzyme inhibition is shown) were derived from a nonlinear regression model (curvefit) based on sigmoidal dose response curve (variable slope) and
computed using Graphpad Prism version 5.02, (Graphpad Software, La Jolla, CA, USA). Data were expressed as mean ± SE.

3.10.2. Cell Culture and Western Blotting

SH-SY5Y human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in 1:1 DMEM:Ham's F12 containing 10% (v/v) fetal bovine serum (HyClone, Logan, UT, USA), 1% penicillin, and 1% streptomycin at a humidified atmosphere with 5% CO₂. The medium was changed every 2 days. For experiments, cells were and grown in 12-well plates until ~80% confluence, serum-deprived for 12 hours, incubated with GSK-3β inhibitors for 1 hour and Aβ25-35 (amyloid beta peptide 25–35, Sigma, St. Louis, MO, USA) for another 6 hours. Cells were rinsed twice with ice-cold PBS and lysed with 1 × SDS loading buffer. Samples were electrophoresed on 10% SDS-polyacrylamide gels, and transferred onto PVDF membranes. The membranes were blocked for 1 h with 5% (w/v) milk, incubated with rabbit anti-Tau [pS396] phospho-specific antibody (Abcam, Cambridge, UK) for 2 h and the anti-rabbit secondary antibody for 1 h. Antigen-antibody complexes were detected by the ECL Kit.

4. Conclusions

In conclusion, a series of novel 3-benzisoxazolyl-4-indolyl-maleimides were synthesized and identified as potent and selective GSK-3β inhibitors. Among them, compound 7j was the most promising GSK-3β inhibitor, with an IC₅₀ of 0.73 nM, which was about 140-fold more potent than staurosporine. Further cell-based functional assays showed that all selected compounds such as 7c, 7f, 7j–l and 7o–q could obviously reduce Tau phosphorylation at 100 nM by inhibiting GSK-3β. Preliminary SAR and molecular modeling studies provided further insights into interactions between GSK-3β and its inhibitors. The results are useful for the design of novel and selective GSK-3β inhibitors. Future progress on related series will be reported in due course.

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Conflict of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are available from the authors.

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