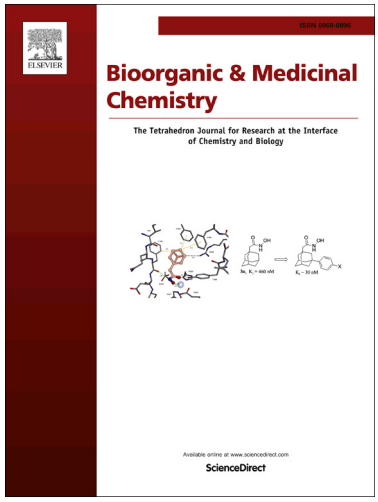

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Design, Synthesis and Biological Evaluation of Bivalent Benzoxazolone and Benzothiazolone Ligands as Potential Anti-inflammatory/Analgesic Agents

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Running title: *Piperazine Dimers Targeting iNOS and NF-κB*

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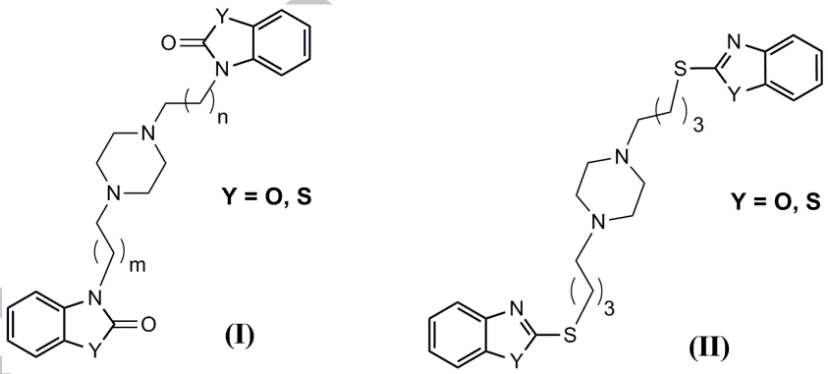
ABSTRACT

Benzoxazolone and benzothiazolone were used as template blocks to develop two series of dimers as anti-inflammatory and analgesic agents based on the concept of bivalent ligands. The first series **(I)** involved varying the carbon chain lengths extending from the piperazine core to the nitrogen atom of the dibenzo[*d*]oxazol-2(3*H*)-one or dibenzo[*d*]thiazol-2(3*H*)-one. The second series **(II)** was designed by changing the attachment point. All compounds were screened for their *in vitro* anti-inflammatory activity in terms of the inhibition of inducible nitric oxide synthase (iNOS) and nuclear factor kappaB (NF-κB). Seventeen compounds inhibited both targets. Eleven of them exhibited IC_{50} values below 3 μM while five compounds showed IC_{50} values of 1 μM or below. Most of the compounds were found to be devoid of cytotoxicity against mammalian kidney and solid tumors cell lines up to 25μg/mL. *In vivo* anti-inflammatory and antinociceptive studies revealed that compounds **3j**, **5t** and **8b** have significant anti-inflammatory and analgesic activity comparable to that of indomethacin and ketorolac, respectively.

Graphical abstract



Keywords:

Anti-inflammatory/analgesic, Nociception, iNOS, NF-κB, benzoxazolone, benzothiazolone, piperazine, dimers

1. Introduction

Inflammation is a multifactorial process caused by various stimuli including physical damage, irradiation, microbial infections and immune reactions.[1] There are several molecular targets recognized from the inflammation cascades that could be antagonized to block the output of these cascades.[2] The major anti-inflammatory targets include cyclooxygenases (COXs), lipoxygenases (LOXs), nitric oxide synthases (NOS) enzymes, nuclear factor kappa B (NF-κB),[3] cytokines, cytokine receptors, tumor necrosis factor (TNF-α), chemokines, interferons (IFNs) and many other targets.[4] Over the past few decades, three isoforms of nitric oxide synthase have been identified and found to catalyze the formation of nitric oxide (NO) from arginine.[5] Of the three isoforms, inducible nitric oxide synthase (iNOS), which is responsible for the overproduction of NO, has been involved in a number of inflammatory diseases such as rheumatoid arthritis, asthma, inflammatory bowel disease and more recently, in neuropathic pain.[6,7] Therefore, selective inhibition of iNOS would be a valid approach and prime target for the reduction of inflammation and alleviation of the associated pain. On the other hand, NF-κB represents a group of structurally related and evolutionarily conserved proteins that are responsible for the regulation of the expression of several genes involved in inflammatory response, including iNOS and dynorphins at the transcriptional level.[8,9] In addition, it has been established that NF-kB signaling pathways promote the gene expression of many proinflammatory cytokines as well as tightly controlling COX-2 expression.[10] Several studies have indicated the contribution of components of the NF-κB signaling pathways in the pathogenesis of chronic inflammatory diseases especially, rheumatic ones.[11] Furthermore, there has been much evidence that a strong relationship exists between the pathological processes of inflammation and cancer.[12] NF-kB was found to be the central molecular link between inflammation and carcinogenesis and its activation plays a pivotal role in growth and progression of inflammation-induced cancer.[13] Moreover, many proinflammatory cytokines and chemokines in the tumor microenvironment which activate the IKKβ-dependent NF-κB signaling pathway have been demonstrated to be associated with cancer development and progression.[14,15] Based on the critical role of the inflammatory microenvironment in progression of several cancer types, it was proposed that targeting the inflammatory components rather than the malignant cell may offer a better therapeutic approach that could prevent the quick mutation and acquired drug resistance.[12,16,17] Numerous reports have demonstrated that NF-κB can stimulate cell proliferation, enhance angiogenesis, suppress apoptosis, and promote metastasis via inducing the expression of diverse target

genes.[18] Thus, it was suggested that the inhibition of NF-κB activation and its upstream signaling pathway may warrant a potential cancer therapy strategy.[18] However, several challenges may counteract the using of NF-kB inhibitors in clinical practice. First, the inhibition of NF-kB may impair immune functions since NF-kB activation is important for maintaining both innate and acquired immunity.[19] Also, the mechanisms that leads to the activation of IKK-β and NF-kB in both malignant and the inflammatory components of cancer need to be more clearly identified and fully understood.[12]

To date, Non-steroidal anti-inflammatory drugs (NSAIDs) continue to be the most commonly utilized medications for inflammation and pain.[20] Concomitantly, NSAIDs are known to have several serious adverse effects such as gastric ulceration, renal injury and recently, cardiotoxicity.[21] Therefore, there remains a substantial need for researchers to find new compounds devoid of these side-effects. In an attempt to overcome these associated liabilities, we herein, tried to introduce iNOS and NF-κB dual inhibitors as a potential strategy for the development of anti-inflammatory/analgesic agents without serious side effects. Our design of these agents was based on the previously reported compound **(I),** S-14080, that has anti-inflammatory and antinociceptive activities superior to acetylsalicylic acid and equivalent to glafenine. Compound (**I**) was found to inhibit the arachidonic acid inflammatory cascade.[22] Moreover, scaffold **(II)** was found to have a significant analgesic activity.[23] From the study of the structural features of the aforementioned compounds, benzoxazolonone and benzothiazolonone heterocycles in addition to the piperazine core are important for both anti-inflammatory and analgesic activities In this context, we decided to incorporate these moieties in one template structure **(III)** utilizing the fragment-based drug design concept (**Fig. 1**). Consequently, to investigate the effect of carbon linker length between the piperazine core and the heterocyclic moiety on the activity, we synthesized two series of dimers, homodimers and heterodimers, and varied the length of carbon chain from two to six carbons.

2 . Results and discussion

2.1. Chemistry

The synthesis of these compounds was facile and straightforward as outlined in **Scheme 1**. The first series was designed as homodimers with equal length carbon chains around piperazine ring. Commercially available 2(3H)-benzoxazolone and 2(3H)-benzothiazolone were N-alkylated with various dibromoalkanes to give intermediates **2a-j.** Then, reaction of two equivalents of **2a-j** with one equivalent of piperazine was performed to give the targeted

compounds **3a-j** that finally separated as hydrochloride salts for biological testing. While the second series was designed as heterodimers where the carbon chains around piperazine ring were different. The series **3a-j** will aid in understanding the structure activity relationship for the inhibition of iNOS and NF-κB activities. The synthesis of these compounds is also outlined in **Scheme 1.** N-alkylation of 2(3H)-benzoxazolone or 2(3H)-benzothiazolone by various dibromoalkanes coupled with an equimolar amount of piperazine afforded intermediates **4a-h**. These intermediates were then treated with bromoalkyl benzoxazolones or bromoalkyl benzthiazolones **2c-j** to give the desired heterodimers **5a-t**. Finally, dihydrochloride salts were formed for biological testing.

To explore the role of the heterocycle attachment position, a series of S-alkyltaed compounds were designed. **Scheme 2** outlines the synthesis of the S-alkylated compounds wherein dibromobutane was reacted with the previously thionated 2(3H)-benzoxazolone or 2(3H)-benzothiazolone with Lawesson's reagent to give intermediates **7a-b**. The dibromobutane was directed to the more reactive 2-thiocarbonyl. Subsequently, intermediates **7a-b** were utilized to alkylate the piperazine ring resulting in compounds **8a-b**.

2.2. Pharmacology

All the newly synthesized compounds, **3a-3j**, **5a–5t** and **8a-8b** were screened through cellular assays for their anti-inflammatory/analgesic potential based on their inhibitory activities on two targets; iNOS and NF-κB. The results are expressed in terms of IC_{50} values (the concentration that caused a 50% inhibition) and presented in **Table 1**. In addition, the cytotoxicity towards a panel of mammalian cell lines was also determined. Based on the *in vitro* results, three compounds **3j**, **5t** and **8b** were selected for *in vivo* efficacy study. The acetic acid induced-writhing test was used for testing the analgesic activity and the carrageenan-induced assay was used for assessment of the anti-inflammatory activity.

2.2.1. iNOS enzyme inhibitory assay

The iNOS inhibitory assay was performed in LPS-induced mouse macrophages (RAW264.7) where the concentration of (NO) was determined by measuring the level of nitrite in the cell culture supernatant using *Griess* reagent.[24] L-N-monomethyl Arginine (L-NMMA) was used as positive control. The homodimers **3h** and **3j** which contain benzothiazolone nucleus and the longest alkyl chains displayed the most potent inhibition of iNOS in this series with IC_{50} values of 0.41 and 0.28 μM, respectively (**Table 1**). On the other hand, the corresponding

benzoxazolone dimers **3g** and **3i** were not as potent ($IC_{50,}$ 4.4 μM and 4.2 μM) although they have equal alkyl chains. The rest of the homodimer series, **3a-3f**, with shorter alkyl chains did not show any activity with the exception of **3d** ($IC_{50,}$ 4.6 μM). Subsequently, structure-activity relationship studies were performed to deduce how the variation of the alky chain length could affect the activity. In this regard, a series of heterodimers were synthesized and evaluated for their potential to inhibit iNOS activity. Out of the benzothiazolone heterodimers, two compounds **5r** and **5t** were the most active with IC_{50} values of 0.51 and 0.29 μM, respectively, while others (**5h**, **5l**, **5n** and **5p**) with shorter alkyl chains were not as effective (IC_{50} values in the range of 1.44 - 3.43 μM). On the other hand, the benzoxazolone dimers **5q** and **5s** with the same alkyl chains length showed activity with IC_{50} values of 1.77 and 2.9 μM, respectively. However, other heterodimers were found to show weak activity (**5e-5g**, **5j-5k**, **5m**, **5o**) or they were devoid of any activity (**5a-5d** and **5i**). It was worth noting that the difference in attachment point of the alkyl chain to the heterocycle significantly affected the activity. Compounds **8a** and **8b** showed better iNOS inhibitory activities with IC_{50} values 1.12 and 0.41 μM, respectively. On the contrary, compounds **3e** and **3f** with the same alkyl chains length, attached to the nitrogen not to the sulfur atom, displayed no activity.

2.2.2. Inhibition of NF-κB transcriptional activity

The effect of compounds on the transcriptional activity of NF-κB was determined in PMA-induced human chondrosarcoma (SW1353) cells through a reporter gene assay.[25] Parthenolide was used as a positive control. Interestingly, most compounds showing strong inhibition of iNOS also showed a strong inhibition of NF-κB activity. The benzothiazolone derivatives **3h** and **3j** exhibited the highest activity with IC_{50} values of 0.4 μM while, the corresponding benzoxazolone dimer **3g** was not as effective with an IC_{50} value of 3.43 μM, and **3i** was devoid of any activity (**Table 1**). Moreover, heterodimers **5p, 5r** and **5t** with long alkyl chains showed acceptable inhibitory activity with IC_{50} values of 1.18, 0.83 and 1.06 μM respectively. On the other hand, the benzoxazolone dimers **5q** and **5s** were equally active to the corresponding benzothiazolone dimers **5r** and **5t** with IC_{50} values 1.24 and 1.40 μM, respectively. Nevertheless, compounds **5h, 5j** and **5n** were moderately active while most of the corresponding benzoxazolone dimers were devoid of activity except compound **5g** which displayed a weak activity with an IC_{50} value of 3.07 μM.

It should be mentioned that, changing the attachment point of the alkyl chains to the heterocycle increases the activity similar to its effect on the iNOS inhibition. Compounds **8a** and **8b** more potent than the corresponding dimers **3e** and **3f** where their alkyl chains attached directly to the nitrogen atom of the heterocycle rather than the sulfur atom. In addition, it was noted that the benzothiazolone dimer **8b** has higher inhibitory activity than the benzoxazolone dimer **8a** with IC_{50} values of 0.34 and 1.40 μM, respectively.

These findings indicate that the heterodimers were, in general, more active than homodimers. Furthermore, the benzothiazolone dimers that have long alkyl chains were more potent inhibitors of both iNOS and NF-κB than the corresponding benzoxazolone dimers. None of the compounds showed inhibition of Sp-1 activity which confirms that their activity towards NF-κB is specific and is not related to cytotoxicity.

2.2.3. Acetic acid-induced writhing test

Two compounds, **3j** and **8b**, which exhibited the highest inhibitory activities *in vitro* against NF-κB and iNOS targets were selected to be evaluated for antinociceptive potential *in vivo* using the well-known acetic acid-induced writhing assay. The effects of these ligands on the acetic acid abdominal writhing assay are summarized in **Fig. 2.** Indomethacin was used as a standard control for this assay. In vehicle treated mice, acetic acid produced robust writhing responses during the observation period. Mice that received indomethacin displayed fewer writhing responses. The test compounds **3j** and **8b** produced significant reduction in writhing responses. However, the results revealed that the effect of the test compounds on the number of writhing was markedly increased with the dose elevating from 3 mg/kg to 10 mg/kg.

2.2.4. Carrageenan-induced rat paw edema assay

The anti-inflammatory activities of compounds **3j**, **5t** and **8b** were tested using carrageenan-induced rat paw edema assay where ketorolac was used as the reference drug.[26] Mean changes in paw edema thickness of animals pretreated with the test compounds were measured after 1, 2, 3, and 4 hours from induction of inflammation. Data was analyzed by one-way ANOVA, followed by Dunnett's multiple comparisons test (n = 5). All compounds showed significant anti-inflammatory activity comparable to that of ketorolac (**Table 2**). Results revealed that compound **8b** was the most active dimer while compounds **3j** and **5t** showed moderate activities compared to ketorolac. These results verified that the attachment point of the alkyl chain significantly influences the activity *in vivo* as well as *in vitro*. Another observation is the decline in activity of tested compounds after 3 hours. This could

be explained by the expected rapid degradation of test compounds via N-dealkylation or other metabolic pathways.

2.2.5. Cytotoxicity assay

All compounds were evaluated for cytotoxicity against a panel of four human solid tumor cell lines (SK-MEL: malignant-melanoma; KB: oral epidermal carcinoma; BT-549: breast ductal carcinoma, and SK-OV-3: ovary carcinoma) and two kidney cell lines (Vero: monkey kidney fibroblasts and LLC-PK1: pig kidney epithelial cells).[27] Most of the compounds were not cytotoxic up to a concentration of 25 µg/mL and do not seem to have any significant anti-cell proliferative activity towards cancer cells as shown in **Table1**.

2.3. Docking study

In order to understand the structural basis for activity and the putative binding mode of our ligands in the active site of iNOS, the best compounds **3j**, **5t** and **8b** were docked by employing LIGANDFIT embedded in Discovery Studio software.[28] The X-ray structure of iNOS complexed with the selective inhibitor AR-C120011 (pdb code: 3E65) was selected for this approach. All compounds adopted a disposition and orientation similar to that of the selective inhibitor AR-C120011. It has been reported that the correlated side-chain rotations of Gln257, Arg260 and other residues expose a new specificity pocket extending from the active-site heme pocket.[29] This new, larger pocket may explain the accommodation of the iNOS binding site for these bulky dimers and potentially their enhanced inhibitory activity. All docked compounds showed three critical features and interactions within the binding pocket of iNOS: 1) The heterocycle, benzoxazolone or benzothiazolone, made a stacking interaction with heme propionate; 2) The piperazine core was enclosed by a polar cavity consisting of Gln257, Val346, and Arg260 residues forming a network of hydrogen bonds between the piperazine nitrogen atoms and the former amino acids; and 3) an additional hydrogen bond tethered the sulfur or the carbonyl group of the extended inhibitor tails to Asn115 residue, as shown in **Fig. 3**. It could also be appreciated from **Fig. 3(A)** how compound **8b** formed another hydrogen-bonding interaction with NH of Val346. In conclusion, the results indicate contributions from heme stacking, hydrogen bonding and hydrophobic interactions within the active site account for the binding mode of these inhibitors and potentially explain their activity.

3. Conclusion

Two series of benzoxazolone and benzothiazolone dimers were synthesized and evaluated *in vitro* and *in vivo* for their anti-inflammatory and analgesic activities. The first series was designed by varying the carbon chain length extending from the piperazine core to the nitrogen atom of the dibenzo[*d*]oxazol-2(3*H*)-one or dibenzo[*d*]thiazol-2(3*H*)-one. The second series was designed by changing the attachment point. Compounds **3j**, **5t** and **8b** were the most potent in inhibiting iNOS activity with IC_{50} values of 0.28, 0.29 and 0.41 μM respectively. On the other hand, compounds **3h**, **3j** and **8b** were found to be the most potent in inhibiting NF-κB activity with IC_{50} values 0.43, 0.41 and 0.34 μM respectively. Based on the overall results, we found that heterodimers with longer alkyl chains around the piperazine core were more active than homodimers. Moreover, the attachment point on the heterocycle is important and significantly affects the activity. Based on the potent *in vitro* activity towards both iNOS and NF-κB, three compounds were selected for assessing their *in vivo* anti-inflammatory/analgesic potential. Compound **8b** exhibited promising *in vivo* activity which was comparable to indomethacin and ketorolac. The docking simulation was carried out for some active dimers to determine the probable binding modes and poses. The results indicated that these active compounds fit nicely into the iNOS binding site, resulting in a stable complex and acting as potential inhibitors of this enzyme.

4. Experimental protocols

4.1. Chemistry

Reagents and starting materials were obtained from commercial suppliers and were used without purification. Precoated silica gel GF Uniplates from Analtech were used for thin-layer chromatography (TLC). Column chromatography was done on silica gel 60 (Sorbent technologies). ^{1}H and ^{13}C NMR spectra were obtained on a Bruker APX400 at 400 and 100 MHz, respectively. The high resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-Tof Micro mass spectrometer with a lock of spray source. The mass spectra (MS) were recorded on a Waters Acquity Ultra Performance LC with ZQ detector in ESI mode. Elemental analysis (C, H, N) were recorded on an elemental analyzer, Perkin-Elmer CHN/SO series II Analyzer. Chemical names were generated using ChemDraw Ultra (CambridgeSoft, version 12.0).

General Procedure A. Synthesis of bromoalkyl benzo[d]oxazol-2(3H)-one and bromoalkyl benzo[d] thiazol-2(3H)-one derivatives. 3-(4-bromobutyl)benzo[d]oxazol-2(3H)-one **(2e).**

K_2CO_3 (9.2 g, 66.6 mmol) and 1,4-dibromobutane (21.0 mL, 177.6 mmol) were added, under stirring, to a solution of benzo[*d*]oxazol-2(3*H*)-one (3.0 g, 22.2 mmol) in anhydrous DMF (30 mL). The reaction mixture was heated at 60 °C for 3 h. After cooling, the reaction mixture was poured into 100 mL water and extracted with ethyl acetate (3 x 70 mL). The combined organic layers were washed with saturated aqueous NaCl and dried over sodium sulfate. The solvent was removed in vacuo, and the residue was purified by flash column chromatography (SiO_2) using hexane/ ethyl acetate (8: 2) as eluent to give 3.6 g (62%) of **2e** as a white solid. ^{1}H NMR ($CDCl_3$): δ 7.30 – 7.05 (m, 3H), 7.00 (d, *J* = 7.4, 1H), 3.86 (t, *J* = 5.1, 2H), 3.44 (t, *J* = 10.6, 2H), 1.95 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 154.54, 142.67, 130.94, 123.89, 122.49, 110.09, 108.25, 41.28, 32.74, 29.41, 26.31. MS (EI) *m/z* 292 (M^++23).

3-(2-bromoethyl)benzo[d]oxazol-2(3H)-one ***(2a).*** General Procedure A, yield 58%, yellow oil.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 6.79 (m, 4H), 4.21 (t, *J* = 6.2, 2H), 3.67 (t, *J* = 6.1, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.20, 142.54, 130.83, 123.96, 122.75, 110.17, 108.54, 43.88, 27.73. MS (EI) *m/z* 242 (M^++1).

3-(2-bromoethyl)benzo[d]thaizol-2(3H)-one ***(2b).*** General Procedure A, yield 57%, white solid.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 – 6.99 (m, 4H), 4.37 (t, *J* = 6.2, 2H), 3.82 (t, *J* = 6.1, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.07, 154.40, 142.70, 135.83, 134.62, 122.03, 120.40, 55.74, 39.59. MS (EI) *m/z* 258 (M^++1).

3-(3-bromopropyl)benzo[d]oxazol-2(3H)-one ***(2c).*** General Procedure A, yield 59%, yellow oil.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 6.87 (m, 4H), 4.00 (t, *J* = 6.8, 2H), 3.46 (t, *J* = 6.3, 2H), 2.94 – 1.89 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.45, 142.65, 131.13, 123.97, 122.59, 110.14, 108.27, 40.61, 30.75, 29.87. MS (EI) *m/z* 279 (M^++23).

3-(3-bromopropyl)benzo[d]thaizol-2(3H)-one ***(2d).*** General Procedure A, yield 61%, yellow solid.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 – 6.95 (m, 4H), 4.11 (t, *J* = 7.5, 2H), 3.46 (t, *J* = 6.4, 2H), 2.35 – 2.29 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.76, 144.96, 133.45, 126.29, 124.91, 112.46, 110.59, 42.93, 33.06, 32.18. MS (EI) *m/z* 272 (M^++1).

3-(4-bromobutyl)benzo[d]thaizol-2(3H)-one ***(2f).*** General Procedure A, yield 63%, yellow oil.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 – 6.74 (m, 4H), 3.94 (t, *J* = 6.6, 2H), 3.41 (t, *J* = 6.0, 2H), 2.14 – 1.63 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.79, 136.85, 126.44, 123.15, 122.72, 122.66, 110.56, 41.67, 33.07, 29.58, 26.16. MS (EI) *m/z* 308 (M^++23).

3-(5-bromopentyl)benzo[d]oxazol-2(3H)-one ***(2g).*** General Procedure A, yield 65%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 – 6.95 (m, 4H), 3.84 (t, *J* = 7.2, 2H), 3.39 (t, *J* = 6.7,

2H), 1.97 – 1.87 (m, 2H), 1.85-1.78 (m, 2H), 1.63 – 1.45 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.54, 142.68, 131.08, 123.82, 122.39, 110.07, 108.21, 42.00, 33.21, 32.09, 26.96, 25.21. MS (EI) *m/z* 306 (M^++23).

3-(5-bromopentyl)benzo[d]thaizol-2(3H)-one ***(2h).*** General Procedure A, yield 64%, yellow oil.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 – 6.86 (m, 4H), 3.89 (t, *J* = 7.3, 2H), 3.33 (t, *J* = 6.7, 2H), 1.88 – 1.81 (m, 2H), 1.74 – 1.67 (m, 2H), 1.52 – 1.44 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.67, 136.95, 126.37, 123.05, 122.68, 122.66, 110.55, 42.42, 33.48, 32.20, 26.76, 25.32. MS (EI) *m/z* 323 (M^++23).

3-(6-bromohexyl)benzo[d]oxazol-2(3H)-one ***(2i).*** General Procedure A, yield 65%, yellow oil.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 6.73 (m, 4H), 3.82 (t, *J* = 7.2, 2H), 3.38 (t, *J* = 6.7, 2H), 1.95 – 1.66 (m, 4H), 1.66 – 1.23 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.56, 142.67, 131.14, 123.79, 122.33, 110.01, 108.25, 42.10, 33.65, 32.48, 27.66, 27.61, 25.82. MS (EI) *m/z* 298 (M^++1).

3-(6-bromohexyl)benzo[d]thaizol-2(3H)-one ***(2j).*** General Procedure A, yield 63%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 – 6.77 (m, 4H), 3.91 (t, J = 7.2, 2H), 3.36 (t, *J* = 6.7, 2H), 1.94 – 1.60 (m, 4H), 1.56 – 1.24 (m, 4H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 206.96, 198.73, 196.03, 195.20, 195.13, 195.11, 192.07, 174.98, 172.76, 172.45, 171.25, 171.17, 170.79. MS (EI) *m/z* 314 (M^++1).

General Procedure B. Synthesis of piperazine dibenzo[d]oxazol-2(3H)-one Homodimers. 3,3'-(piperazine-1,4-diylbis(butane-4,1-diyl))bis(benzo[d]oxazol-2(3H)-one) ***(3e).*** K_2CO_3 (0.76 g, 5.55 mmol) and piperazine (0.1 g, 0.92 mmol) were added, under stirring, to a solution of **2e** (0.5 g, 1.85 mmol) in anhydrous DMF (10 mL). The reaction mixture was heated at 60 °C for 1.5 h. After cooling, the reaction mixture was poured into 50 mL water and extracted with ethyl acetate (3 x 30 mL). The combined organic layers were washed with saturated aqueous NaCl and dried over sodium sulfate. The solvent was removed in vacuo, and the residue was purified by flash column chromatography (SiO_2) using dichlormethane/ methanol (9.5: 0.5) as eluent to give 0.3 g (70%) of **3e** as a white solid. ^{1}H NMR ($CDCl_3$): δ 7.26-6.96 (m, 8H), 3.82 (t, *J* = 7.0, 4H), 2.39 (s, 8H), 2.37 – 2.29 (m, 4H), 1.78 -1.75 (m, 4H), 1.57-1.52 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 154.51, 142.65, 131.12, 123.73, 122.27, 109.97, 108.30, 57.66, 53.13, 42.08, 25.61, 23.88. HRMS calcd for $C_{26}H_{32}N_4O_4$ $[M+H]^+$ 464.2424, found 465.2509. Anal. calcd for $C_{26}H_{34}Cl_2N_4O_4$: C, 58.10; H, 6.38; N, 10.42. Found: C, 58.34; H, 6.87; N, 10.21.

3,3'-(piperazine-1,4-diylbis(ethane-2,1-diyl))bis(benzo[d]oxazol-2(3H)-one) ***(3a).*** General Procedure B, yield 51%, white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 – 7.00 (m, 8H), 4.06 (t, *J* = 7.1, 4H), 2.72 – 2.62 (t, *J* = 7.2, 4H), 2.56 (s, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.87, 137.08, 126.27, 123.03, 122.75, 122.66, 110.57, 54.85, 53.27, 40.40. HRMS calcd for $C_{22}H_{24}N_4O_4$ $[M+H]^+$ 408.1798, found 409.1891. Anal. calcd for $C_{22}H_{26}Cl_2N_4O_4$: C, 54.89; H, 5.44; N, 11.64. Found: C, 55.14; H, 5.87; N, 11.28.

3,3'-(2,2'-(piperazine-1,4-diyl)bis(ethane-2,1-diyl))dibenzo[d]thaizol-2(3H)-one ***(3b).*** General Procedure B, yield 52%, white solid.^{1}H NMR (400 MHz, DMSO) δ 7.85 – 6.92 (m, 8H), 4.32 (t, *J* = 6.7, 4H), 3.30 – 3.16 (m, 12H). ^{13}C NMR (101 MHz, DMSO) δ 169.53, 136.88, 127.15, 123.82, 123.40, 122.06, 112.00, 52.59, 49.59, 37.54. HRMS calcd for $C_{22}H_{24}N_4O_2S_2$ $[M+H]^+$ 440.1431, found 441.1432. Anal. calcd for $C_{22}H_{26}Cl_2N_4O_2S_2$: C, 51.46; H, 5.10; N, 10.91. Found: C, 51.31; H, 5.47; N, 10.55.

3,3'-(piperazine-1,4-diylbis(propane-3,1-diyl))bis(benzo[d]oxazol-2(3H)-one) ***(3c).*** General Procedure B, yield 53%, white solid. ^{1}H NMR (400 MHz, D_2O) δ 7.27 – 7.03 (m, 8H), 3.86 (t, *J* = 6.5, 4H), 3.19 (s, 8H), 3.00 (t, *J* = 6.8, 4H), 2.07 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.28, 142.41, 131.79, 124.19, 122.40, 109.97, 109.62, 55.25, 52.99, 24.24. HRMS calcd for $C_{24}H_{28}N_4O_4$ $[M+H]^+$ 436.2111, found 437.2173. Anal. calcd for $C_{24}H_{30}Cl_2N_4O_4$: C, 56.58; H, 5.94; N, 11.00. Found: C, 56.91; H, 5.43; N, 11.29.

3,3'-(piperazine-1,4-diylbis(propane-3,1-diyl))bis(benzo[d]thiazol-2(3H)-one) ***(3d).*** General Procedure B, yield 50%, white solid. ^{1}H NMR (400 MHz, DMSO) δ 7.81 – 7.02 (m, 8H), 4.03 (t, *J* = 6.8, 4H), 3.69 – 3.03 (m, 12H), 2.27 – 1.96 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 169.35, 137.18, 127.11, 123.67, 123.36, 122.06, 111.89, 48.47, 22.61, 17.88. HRMS calcd for $C_{24}H_{28}N_4O_2S_2$ $[M+H]^+$ 468.1735, found 469.1736. Anal. calcd for $C_{24}H_{30}Cl_2N_4O_2S_2$: C, 53.23; H, 5.58; N, 10.35. Found: C, 53.59; H, 5.12; N, 10.76.

3,3'-(piperazine-1,4-diylbis(butane-4,1-diyl))bis(benzo[d]thiazol-2(3H)-one) ***(3f).*** General Procedure B, yield 51%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 – 6.46 (m, 8H), 3.92 (t, *J* = 6.9, 4H), 2.42 - 2.32 (m, 12H), 2.02 – 1.60 (m, 4H), 1.56 – 1.54 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.81, 137.05, 126.26, 122.97, 122.73, 122.61, 110.69, 57.64, 53.06, 42.48, 25.37, 23.81. HRMS calcd for $C_{26}H_{32}N_4O_2S_2$ $[M+H]^+$ 496.1967, found 497.2057. Anal. calcd for $C_{26}H_{34}Cl_2N_4O_2S_2$: C, 54.82; H, 6.02; N, 9.84. Found: C, 54.45; H, 6.51; N, 9.65.

3,3'-(piperazine-1,4-diylbis(pentane-5,1-diyl))bis(benzo[d]oxazol-2(3H)-one) ***(3g).*** General Procedure B, yield 53%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 – 6.92 (m, 8H), 3.80 (t, *J* = 7.2, 4H), 2.42 (s, 8H), 2.29 (t, *J* = 7.3, 4H), 1.86 – 1.71 (m, 4H), 1.56 -1.48 (m, 4H), 1.44 – 1.31 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.55, 142.67, 131.17, 123.73, 122.27, 110.01, 108.23, 58.30, 53.19, 42.21, 27.64, 26.38, 24.63. HRMS calcd for $C_{28}H_{36}N_4O_4$ $[M+H]^+$ 492.2737, found 493.2814. Anal. calcd for $C_{28}H_{38}Cl_2N_4O_4$: C, 59.47; H, 6.77; N, 9.91. Found: C, 59.34; H, 6.97; N, 10.33.

3,3'-(piperazine-1,4-diylbis(pentane-5,1-diyl))bis(benzo[d]thiazol-2(3H)-one) ***(3h).*** General Procedure B, yield 52%, yellow oil. ^{1}H NMR (400 MHz, D_2O) δ 8.04 – 6.96 (m, 8H), 4.24 (t, *J* = 6.9, 4H), 3.83 (s, 8H), 3.52 – 3.36 (m, 4H), 2.14 – 1.91 (m, 8H), 1.65 – 1.58 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.45, 143.57, 132.07, 124.63, 123.17, 110.90, 109.13, 59.20, 54.09, 43.11, 28.54, 27.28, 25.53. HRMS calcd for $C_{28}H_{36}N_4O_2S_2$ $[M+H]^+$ 524.2280, found 525.2373. Anal. calcd for $C_{28}H_{38}Cl_2N_4O_2S_2$: C, 56.27; H, 6.41; N, 9.37. Found: C, 56.62; H, 6.81; N, 9.26.

3,3'-(piperazine-1,4-diylbis(hexane-6,1-diyl))bis(benzo[d]oxazol-2(3H)-one) ***(3i).*** General Procedure B, yield 52%, yellow oil.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 – 6.95 (m, 4H), 3.80 (t, *J* = 7.2, 2H), 2.45 (s, 4H), 2.30 (t, *J* = 7.3, 2H), 1.80 – 1.73 (m, 2H), 1.51 -1.44 (m, 2H), 1.42 – 1.34 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.56, 142.68, 131.20, 123.72, 122.26, 110.00, 108.24, 58.45, 53.07, 42.22, 27.68, 27.08, 26.58, 26.56. HRMS calcd for $C_{30}H_{40}N_4O_4$ $[M+H]^+$ 520.3050, found 521.3132. Anal. calcd for $C_{30}H_{42}Cl_2N_4O_4$: C, 60.70; H, 7.13; N, 9.44. Found: C, 60.43; H, 7.17; N, 9.55.

3,3'-(piperazine-1,4-diylbis(hexane-6,1-diyl))bis(benzo[d]thiaazol-2(3H)-one) ***(3j).*** General Procedure B, yield 53%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 – 6.71 (m, 8H), 3.89 (t, *J* = 6.8, 4H), 2.84 – 1.99 (m, 12H), 1.70 (t, *J* = 6.2, 4H), 1.45 – 1.33 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.76, 137.09, 126.25, 122.93, 122.75, 122.62, 110.56, 58.44, 53.02, 42.70, 27.50, 27.14, 26.66, 26.57. HRMS calcd for $C_{30}H_{40}N_4O_2S_2$ $[M+H]^+$ 552.2593, found 553.2695. Anal. calcd for $C_{30}H_{42}Cl_2N_4O_2S_2$: C, 57.59; H, 6.77; N, 8.95. Found: C, 57.14; H, 6.28; N, 9.41.

General Procedure C. Piperazine benzo[d]oxazol-2(3H)-one derivatives. 3-(4-(piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one ***(4e)***. K_2CO_3 (2.3 g, 16.71 mmol) and piperazine (2.4 g, 27.85 mmol) were added, under stirring, to a solution of **2e** (1.5 g, 5.57 mmol) in anhydrous DMF (20 mL). The reaction mixture was heated at 60 °C for 0.5 h. After cooling, the reaction

mixture was poured into 60 mL water and extracted with dichloromethane (3 x 50 mL). The combined organic layers were washed with saturated aqueous NaCl and dried over sodium sulfate. The solvent was removed in vacuo, and the residue was purified by flash column chromatography (SiO_2) using dichloromethane/ methanol: NH_4OH (9: 1) as eluent to give 1.2 g (78%) of **4e** as a white solid. ^{1}H NMR (D_2O): δ 7.16 – 7.06 (m, 4H), 3.72 (t, *J* = 6.0, 2H), 3.62 (s, 8H), 3.27 (t, *J* = 6.3, 2H), 1.76 -1.68 (m, 4H). ^{13}C NMR (101 MHz, D_2O) δ 156.03, 142.10, 130.26, 124.44, 123.05, 110.02, 109.36, 56.63, 48.47, 41.27, 40.81, 24.17, 20.65. MS (EI) *m/z* 276 (M^++1).

3-(2-(piperazin-1-yl)ethyl)benzo[d]oxazol-2(3H)-one ***(4a).*** General Procedure C, yield 67%, white solid. ^{1}H NMR (400 MHz, D_2O) δ 7.31 – 7.13 (m, 4H), 4.30 (t, *J* = 5.9, 2H), 3.77 (s, 4H), 3.72 (t, *J* = 6.3, 2H), 3.64 (s, 4H). ^{13}C NMR (101 MHz, D_2O) δ 156.20, 142.47, 129.53, 124.69, 123.70, 110.46, 109.22, 54.25, 48.86, 40.72, 36.50. MS (EI) *m/z* 248 (M^++1).

3-(2-(piperazin-1-yl)ethyl)benzo[d]thaizol-2(3H)-one ***(4b).*** General Procedure C, yield 68%, white solid ^{1}H NMR (400 MHz, D_2O) δ 7.69 – 6.78 (m, 4H), 4.29 (t, *J* = 6.0, 2H), 3.68 (s, 4H), 3.64 – 3.40 (m, 6H). ^{13}C NMR (101 MHz, D_2O) δ 173.78, 135.44, 127.08, 124.30, 123.12, 122.16, 111.08, 54.24, 48.90, 40.69, 36.85. MS (EI) *m/z* 264 (M^++1).

3-(3-(piperazin-1-yl)propyl)benzo[d]oxazol-2(3H)-one ***(4c).*** General Procedure C, yield 66%, white solid ^{1}H NMR (400 MHz, D_2O) δ 7.27 – 7.01 (m, 4H), 3.88 (t, *J* = 6.7, 2H), 3.62 (s, 8H), 3.37 (t, *J* = 6.5, 2H), 2.35 – 2.13 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 156.00, 142.21, 130.12, 124.54, 123.26, 110.19, 109.23, 54.50, 48.65, 40.84, 39.08, 22.26. MS (EI) *m/z* 262 (M^++1).

3-(3-(piperazin-1-yl)propyl)benzo[d]thiazol-2(3H)-one ***(4d).*** General Procedure C, yield 63%, white solid ^{1}H NMR (400 MHz, D_2O) δ 7.17 – 7.06 (m, 4H), 3.72 (t, *J* = 6.8, 2H), 3.62 (s, 8H), 3.29 (t, *J* = 6.5, 2H), 1.79 – 1.76 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 156.03, 142.10, 130.26, 124.44, 123.05, 110.02, 109.36, 56.63, 48.47, 41.27, 40.81, 24.17. MS (EI) *m/z* 277 (M^+).

3-(4-(piperazin-1-yl)butyl)benzo[d]thaizol-2(3H)-one ***(4f).*** General Procedure C, yield 65%, yellow oil ^{1}H NMR (400 MHz, D_2O) δ 7.67 – 6.90 (m, 4H), 3.95 (t, *J* = 5.9, 2H), 3.46 – 3.38 (m, 8H), 3.20 – 2.86 (m, 2H), 1.87 – 1.58 (m, 2H), 1.22 – 1.17 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 156.91, 142.99, 131.15, 125.33, 123.93, 110.91, 110.25, 57.52, 49.36, 42.16, 41.70, 25.06, 21.54. MS (EI) *m/z* 292 (M^++1).

3-(5-(piperazin-1-yl)pentyl)benzo[d]oxazol-2(3H)-one ***(4g).*** General Procedure C, yield 67%, white solid ^{1}H NMR (400 MHz, D_2O) δ 7.22 - 7.11 (m, 4H), 3.75 (t, *J* = 6.8, 2H), 3.61 (s, 8H), 3.22 (t, *J* = 6.5, 2H), 1.86 – 1.63 (m, 4H), 1.41 -1.33 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 156.26, 142.21, 130.51, 124.40, 123.00, 110.05, 109.50, 57.07, 48.40, 41.70, 40.79, 26.45, 22.85, 22.81. MS (EI) *m/z* 290 (M^++1).

3-(5-(piperazin-1-yl)pentyl)benzo[d]thaizol-2(3H)-one ***(4h).*** General Procedure C, yield 66%, white solid ^{1}H NMR (400 MHz, D_2O) δ 7.89 – 6.53 (m, 4H), 3.79 – 3.31 (m, 10H), 3.13 (t, *J* = 7.3, 2H), 1.72 – 1.57 (m, 2H), 1.54 – 1.52 (m, 2H), 1.24 – 1.23 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 136.36, 136.32, 126.81, 123.73, 122.64, 121.80, 111.64, 100.00, 56.98, 48.32, 42.16, 40.71, 26.29, 22.81. MS (EI) *m/z* 306 (M^++1).

General Procedure D. Synthesis of piperazine dibenzo[d]oxazol-2(3H)-one Heterodimmers.

3-(5-(4-(4-(2-oxobenzo[d]oxazol-3(2H)-yl)butyl)piperazin-1-yl)pentyl)benzo[d]oxazol-2(3H)-one ***(5o).*** K_2CO_3 (0.75 g, 5.45 mmol) and **4e** (0.5 g, 1.82 mmol) were added, under stirring, to a solution of **2g** (0.49 g, 1.82 mmol) in anhydrous DMF (20 mL). The reaction mixture was heated at 60 °C for 2 h. After cooling, the reaction mixture was poured into 60 mL water and extracted with ethyl acetate (3 x 40 mL). The combined organic layers were washed with saturated aqueous NaCl and dried over sodium sulfate. The solvent was removed in vacuo, and the residue was purified by flash column chromatography (SiO_2) using dichlormethane/ methanol (9.5: 0.5) as eluent to give 0.58 g (73%) of **5o** as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 7.42 – 6.82 (m, 8H), 3.74 (t, *J* = 6.7, 2H), 3.69 (t, *J* = 6.9, 2H), 3.57 (s, 8H), 3.22 (t, *J* = 7.8, 2H), 3.19 – 3.12 (m, 2H), 1.84 – 1.57 (m, 8H), 1.34 – 1.27 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 156.21, 156.20, 142.17, 142.13, 130.41, 130.28, 124.37, 124.32, 123.03, 122.92, 110.04, 109.97, 109.40, 109.30, 56.65, 56.20, 48.60, 48.56, 41.59, 41.14, 26.38, 24.07, 22.88, 22.71, 20.66. HRMS calcd for $C_{27}H_{34}N_4O_4$ $[M+H]^+$ 478.2580, found 479.3266. Anal. calcd for $C_{27}H_{36}Cl_2N_4O_4$: C, 58.80; H, 6.58; N, 10.16. Found: C, 58.47; H, 6.13; N, 10.39.

3-(3-(4-(2-(2-oxobenzo[d]oxazol-3(2H)-yl)ethyl)piperazin-1-yl)propyl)benzo[d]oxazol-2(3H)-one ***(5a).*** General Procedure D, yield 67%, white solid. ^{1}H NMR (400 MHz, D_2O) δ 7.36 – 7.10 (m, 8H), 4.25 (t, *J* = 5.9, 2H), 3.93 (t, *J* = 6.6, 2H), 3.67 – 3.46 (m, 10H), 3.37 – 3.26 (m, 2H), 2.29 – 2.11 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 156.40, 156.28, 142.53, 130.27, 124.59, 124.50, 123.58, 123.28, 110.44, 110.30, 109.18, 109.16, 54.10, 53.91, 49.35, 49.09, 39.01, 22.27. HRMS calcd for $C_{23}H_{26}N_4O_4$ $[M+H]^+$ 422.1954, found 423.2025. Anal. calcd for $C_{23}H_{28}Cl_2N_4O_4$: C, 55.76; H, 5.70; N, 11.31. Found: C, 56.14; H, 5.24; N, 11.01.

3-(3-(4-(2-(2-oxobenzo[d]thiazol-3(2H)-yl)ethyl)piperazin-1-yl)propyl)benzo[d]thiazol-2(3H)-one ***(5b).*** General Procedure D, yield 63%, white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 – 6.87 (m, 8H), 3.97 – 3.79 (m, 4H), 2.66 (t, *J* = 6.5, 2H), 2.46 – 2.32 (m, 10 H), 1.95 – 1.82 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.55, 142.63, 142.60, 131.43, 131.17, 123.69, 123.67, 122.26, 122.17, 109.91, 109.86, 108.47, 108.44, 55.14, 54.93, 53.15, 52.95, 40.38, 39.81, 24.58. HRMS calcd for $C_{23}H_{26}N_4O_2S_2$ $[M+H]^+$ 454.1497, found 455.1564. Anal. calcd for $C_{23}H_{28}Cl_2N_4O_2S_2$: C, 52.37; H, 5.35; N, 10.62. Found: C, 52.59; H, 5.12; N, 10.11.

3-(2-(4-(4-(2-oxobenzo[d]oxazol-3(2H)-yl)butyl)piperazin-1-yl)ethyl)benzo[d]oxazol-2(3H)-one ***(5c).*** General Procedure D, yield 64%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 – 6.79 (m, 8H), 3.91 (t, *J* = 6.4, 2H), 3.82 (t, *J* = 7.0, 2H), 2.68 (t, *J* = 6.4, 2H), 2.52 (s, 4H), 2.39 – 2.32 (m, 6H), 1.88 – 1.67 (m, 2H), 1.63 – 1.46 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.59, 154.54, 142.67, 131.20, 131.11, 123.74, 123.68, 122.30, 122.28, 110.00, 109.96, 108.44, 108.29, 57.55, 55.18, 53.20, 53.02, 42.08, 39.85, 25.60, 23.85. HRMS calcd for $C_{24}H_{28}N_4O_4$ $[M+H]^+$ 436.2111, found 437.2196. Anal. calcd for $C_{24}H_{30}Cl_2N_4O_4$: C, 56.58; H, 5.94; N, 11.00. Found: C, 56.90; H, 5.88; N, 10.91.

3-(2-(4-(4-(2-oxobenzo[d]thiazol-3(2H)-yl)butyl)piperazin-1-yl)ethyl)benzo[d]thiazol-2(3H)-one ***(5d).*** General Procedure D, yield 61%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 – 6.98 (m, 8H), 4.06 (t, *J* = 7.2, 2H), 3.96 (t, *J* = 7.4, 2H), 2.71 – 2.63 (m, 2H), 2.57 (s, 4H), 2.45 (s, 4H), 2.41 – 2.34 (m, 2H), 1.80 -1.73 (m, 2H), 1.61 - 1.54 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.84, 137.10, 137.09, 126.25, 123.01, 122.97, 122.82, 122.77, 122.65, 122.64, 110.66, 110.56, 57.58, 54.92, 53.31, 53.03, 42.51, 40.42, 29.68, 25.38, 23.82. HRMS calcd for $C_{24}H_{28}N_4O_2S_2$ $[M+H]^+$ 468.1654, found 469.1735. Anal. calcd for $C_{24}H_{30}Cl_2N_4O_2S_2$: C, 53.23; H, 5.58; N, 10.35. Found: C, 53.31; H, 5.17; N, 10.83.

3-(5-(4-(2-(2-oxobenzo[d]oxazol-3(2H)-yl)ethyl)piperazin-1-yl)pentyl)benzo[d]oxazol-2(3H)-one ***(5e).*** General Procedure D, yield 62%, yellow oil. ^{1}H NMR (400 MHz, D_2O) δ 7.57 – 7.43 (m, 8H), 4.53 (t, *J* = 6.0, 2H), 4.08 (t, *J* = 6.8, 2H), 3.91 – 3.75 (m, 10H), 3.51 – 3.36 (m, 2H), 2.15 – 1.87 (m, 4H), 1.66 – 1.59 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 165.86, 142.69, 131.02, 130.10, 124.95, 124.70, 123.93, 123.31, 110.79, 110.46, 109.84, 109.52, 99.99, 56.99, 54.19, 49.35, 49.28, 41.98, 37.36, 34.96, 26.58, 23.04. HRMS calcd for $C_{25}H_{30}N_4O_4$ $[M+H]^+$ 450.2267, found 451.2339. Anal. calcd for $C_{25}H_{32}Cl_2N_4O_4$: C, 57.36; H, 6.16; N, 10.70. Found: C, 57.14; H, 5.94; N, 10.26.

3-(5-(4-(2-(2-oxobenzo[d]thiazol-3(2H)-yl)ethyl)piperazin-1-yl)pentyl)benzo[d]thiazol-2(3H)-one ***(5f).*** General Procedure D, yield 59%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 – 6.89 (m, 8H), 4.04 (t, *J* = 7.2, 2H), 3.92 (t, *J* = 7.4, 2H), 2.64 (t, *J* = 7.1, 2H), 2.56 (s, 4H), 2.42 (s, 4H), 2.30 (t, *J* = 7.3, 2H), 1.77 – 1.70 (m, 2H), 1.55 – 1.48 (m, 2H), 1.45 – 1.32 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.80, 169.79, 137.09, 126.25, 122.99, 122.95, 122.80, 122.73, 122.64, 110.57, 110.54, 58.23, 54.92, 53.33, 53.12, 42.70, 40.42, 27.45, 26.40, 24.71. HRMS calcd for $C_{25}H_{30}N_4O_2S_2$ $[M+H]^+$ 482.1810, found 483.1908. Anal. calcd for $C_{25}H_{32}Cl_2N_4O_2S_2$: C, 54.05; H, 5.81; N, 10.08. Found: C, 54.39; H, 6.17; N, 9.78.

3-(6-(4-(2-(2-oxobenzo[d]oxazol-3(2H)-yl)ethyl)piperazin-1-yl)hexyl)benzo[d]oxazol-2(3H)-one ***(5g).*** General Procedure D, yield 57%, colorless oil. ^{1}H NMR (400 MHz, D_2O) δ 7.34 – 6.83 (m, 8H), 4.25 (t, *J* = 7.3, 2H), 3.82 – 3.42 (m, 12H), 3.27 – 3.06 (m, 2H), 1.70 - 1.64 (m, 4H), 1.45 – 1.20 (m, 4H). ^{13}C NMR (101 MHz, D_2O) δ 156.34, 156.33, 142.47, 142.22, 130.58, 129.57, 124.59, 124.32, 123.60, 122.91, 110.41, 110.02, 109.52, 109.14, 56.85, 56.75, 53.94, 48.99, 48.72, 45.46, 41.86, 36.77, 36.74, 31.44, 26.58, 23.11, 23.09. HRMS calcd for $C_{26}H_{32}N_4O_4$ $[M+H]^+$ 464.2424, found 465.2507. Anal. calcd for $C_{26}H_{34}Cl_2N_4O_4$: C, 58.10; H, 6.38; N, 10.42. Found: C, 57.88; H, 6.10; N, 10.11.

3-(6-(4-(2-(2-oxobenzo[d]thiazol-3(2H)-yl)ethyl)piperazin-1-yl)hexyl)benzo[d]thiazol-2(3H)-one ***(5h).*** General Procedure D, yield 55%, yellow oil. ^{1}H NMR (400 MHz, DMSO) δ 7.57 – 7.06 (m, 8H), 4.33 – 4.17 (m, 2H), 3.81 (t, *J* = 7.0, 2H), 3.74 – 3.01 (m, 12H), 1.82 – 1.57 (m, 4H), 1.48 – 1.24 (m, 4H). ^{13}C NMR (101 MHz, D_2O) δ 153.69, 153.67, 139.82, 139.57, 127.92, 126.92, 121.94, 121.66, 120.95, 120.25, 107.75, 107.36, 106.87, 106.48, 54.09, 51.28, 46.34, 46.06, 42.80, 39.21, 34.11, 28.78, 23.93, 20.43.. HRMS calcd for $C_{26}H_{32}N_4O_2S_2$ $[M+H]^+$ 496.1967, found 497.2053. Anal. calcd for $C_{26}H_{34}Cl_2N_4O_2S_2$: C, 54.82; H, 6.02; N, 9.84. Found: C, 54.30; H, 5.77; N, 10.23.

3-(3-(4-(4-(2-oxobenzo[d]oxazol-3(2H)-yl)butyl)piperazin-1-yl)propyl)benzo[d]oxazol-2(3H)-one ***(5i).*** General Procedure D, yield 53%, colorless oil. ^{1}H NMR (400 MHz, D_2O) δ 7.44 - 7.34 (m, 8H), 4.12 (t, *J* = 6.7, 2H), 4.02 (t, *J* = 6.9, 2H), 3.88 (s, 8H), 3.66 – 3.56 (m, 2H), 3.52 (t, *J* = 7.5, 2H), 2.59 – 2.35 (m, 2H), 2.01-2.00 (m, 4H). ^{13}C NMR (101 MHz, D_2O) δ 142.56, 142.55, 124.81, 124.71, 123.54, 123.34, 122.83, 110.52, 110.50, 110.40, 110.37, 109.66, 109.64, 109.47, 56.58, 54.49, 49.01, 48.84, 41.49, 39.31, 24.33, 22.51, 20.91. HRMS calcd for $C_{23}H_{26}N_4O_4$ $[M+H]^+$ 422.1954, found 423.2025. HRMS calcd for $C_{25}H_{30}N_4O_4$ $[M+H]^+$ 450.2267, found 451.2359. Anal. calcd for $C_{25}H_{32}Cl_2N_4O_4$: C, 57.36; H, 6.16; N, 10.70. Found: C, 57.01; H, 5.84; N, 10.31.

*3-(3-(4-(4-(2-oxobenzo[d]thiazol-3(2H)-yl)butyl)piperazin-1-yl)propyl)benzo[d]thiazol-2 (3H)-one (**5j**).* General Procedure D, yield 52%, yellow oil. ^{1}H NMR (400 MHz, D_2O) δ 7.79 – 7.44 (m, 8H), 4.28 (t, *J* = 6.6, 2H), 4.20 (t, *J* = 6.2, 2H), 3.76 (s, 8H), 3.50 – 3.44 (m, 4H), 2.50 – 2.32 (m, 2H), 2.09 – 1.87 (m, 4H). ^{13}C NMR (101 MHz, D_2O) δ 173.23, 173.17, 136.39, 136.16, 126.98, 126.87, 124.03, 123.89, 122.97, 122.84, 122.06, 122.04, 111.64, 111.35, 56.19, 54.20, 48.74, 48.69, 41.58, 39.43, 23.83, 22.21, 20.58. HRMS calcd for $C_{25}H_{30}N_4O_2S_2$ $[M+H]^+$ 482.1810, found 483.1900. Anal. calcd for $C_{25}H_{32}Cl_2N_4O_4S_2$: C, 54.05; H, 5.81; N, 10.08. Found: C, 54.14; H, 6.12; N, 10.45.

*3-(3-(4-(5-(2-oxobenzo[d]oxazol-3(2H)-yl)pentyl)piperazin-1-yl)propyl)benzo[d]oxazol-2 (3H)-one (**5k**).* General Procedure D, yield 53%, yellow oil. ^{1}H NMR (400 MHz, DMSO) δ 7.85 – 6.76 (m, 8H), 4.02 (t, *J* = 6.3, 2H), 3.93 (t, *J* = 6.6, 2H), 3.68 – 3.08 (m, 12H), 2.11 (m, 2H), 1.73 – 1.64 (m, 4H), 1.33 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.95, 158.87, 126.46, 126.21, 115.40, 115.33, 112.08, 112.04, 111.88, 111.79, 111.76, 111.65, 99.93, 99.68, 47.44, 44.22, 42.35, 42.19, 31.82, 30.09, 16.59, 15.55, 13.96, 13.86. HRMS calcd for $C_{26}H_{32}N_4O_4$ $[M+H]^+$ 464.2424, found 465.2491. Anal. calcd for $C_{26}H_{34}Cl_2N_4O_4$: C, 58.10; H, 6.38; N, 10.42. Found: C, 58.65; H, 6.05; N, 9.97.

*3-(3-(4-(5-(2-oxobenzo[d]thiazol-3(2H)-yl)pentyl)piperazin-1-yl)propyl)benzo[d]thiazol-2 (3H)-one (**5l**).* General Procedure D, yield 51%, colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 – 6.82 (m, 8H), 3.95 (t, *J* = 6.9, 2H), 3.88 (t, *J* = 7.1, 2H), 2.72 – 2.13 (m, 12H), 1.90 - 1.82 (m, 2H), 1.74 – 1.67 (m, 2H), 1.62 – 1.44 (m, 2H), 1.39 – 1.32 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.80, 169.72, 137.31, 137.06, 126.25, 126.18, 122.93, 122.89, 122.73, 122.64, 122.61, 122.51, 110.78, 110.54, 58.29, 55.07, 53.20, 53.04, 42.67, 40.94, 27.44, 26.41, 24.81, 24.71. HRMS calcd for $C_{26}H_{32}N_4O_2S_2$ $[M+H]^+$ 496.1967, found 497.2055. Anal. calcd for $C_{26}H_{34}Cl_2N_4O_2S_2$: C, 54.82; H, 6.02; N, 9.84. Found: C, 55.19; H, 5.94; N, 10.11.

*3-(3-(4-(6-(2-oxobenzo[d]oxazol-3(2H)-yl)hexyl)piperazin-1-yl)propyl)benzo[d]oxazol-2(3H)-one (**5m**).* General Procedure D, yield 56%, yellow oil. ^{1}H NMR (400 MHz, DMSO) δ 7.85 – 6.76 (m, 8H), 4.02 (t, *J* = 6.3, 2H), 3.93 (t, *J* = 6.6, 2H), 3.67 – 3.04 (m, 12H), 2.17 – 2.05 (m, 2H), 1.69 – 1.61 (m, 4H), 1.33 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.67, 165.59, 133.29, 133.06, 122.10, 122.03, 118.77, 118.75, 118.72, 118.64, 118.50, 118.40, 106.64, 106.40, 54.36, 50.99, 49.10, 49.04, 48.96, 38.62, 36.89, 23.41, 23.04, 22.56, 22.53, 20.72.HRMS calcd for $C_{27}H_{34}N_4O_4$ $[M+H]^+$ 478.2580, found 479.2675. Anal. calcd for $C_{27}H_{36}Cl_2N_4O_4$: C, 58.80; H, 6.58; N, 10.16. Found: C, 58.48; H, 6.13; N, 9.85.

*3-(3-(4-(6-(2-oxobenzo[d]thiazol-3(2H)-yl)hexyl)piperazin-1-yl)propyl)benzo[d]thiazol-2(3H)-one (**5n**).* General Procedure D, yield 49%, colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 – 6.94 (m, 8H), 3.98 (t, *J* = 7.0, 2H), 3.90 (t, *J* = 7.1, 2H), 2.66 – 2.13 (m, 12H), 1.92 – 1.85 (m, 2H), 1.76 – 1.68 (m, 2H), 1.62 – 1.27 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.78, 169.69, 137.39, 137.16, 126.20, 126.14, 122.88, 122.85, 122.83, 122.74, 122.66, 122.60, 122.50, 110.75, 110.51, 58.46, 55.10, 53.21, 53.06, 42.73, 40.99, 27.51, 27.15, 26.67, 26.64, 24.83. HRMS calcd for $C_{27}H_{34}N_4O_2S_2$ $[M+H]^+$ 510.2123, found 511.2226. Anal. calcd for $C_{27}H_{36}Cl_2N_4O_2S_2$: C, 55.56; H, 6.22; N, 9.60. Found: C, 55.19; H, 6.57; N, 10.03.

*3-(5-(4-(4-(2-oxobenzo[d]thiazol-3(2H)-yl)butyl)piperazin-1-yl)pentyl)benzo[d]thiazol-2(3H)-one (**5p**).* General Procedure D, yield 54%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 – 6.85 (m, 8H), 3.94 – 3.88 (m, 4H), 2.64 – 2.19 (m, 12H), 1.74 – 1.69 (m, 4H), 1.63 – 1.44 (m, 4H), 1.40 – 1.34 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.71, 169.68, 137.13, 126.21, 122.89, 122.80, 122.78, 122.60, 122.57, 110.66, 110.50, 58.29, 57.61, 53.20, 53.10, 42.70, 42.52, 27.42, 26.40, 25.38, 24.72, 23.85. HRMS calcd for $C_{27}H_{34}N_4O_2S_2$ $[M+H]^+$ 511.2123, found 511.2203. Anal. calcd for $C_{27}H_{36}Cl_2N_4O_2S_2$: C, 55.56; H, 6.22; N, 9.60. Found: C, 56.14; H, 6.17; N, 9.69.

*3-(6-(4-(4-(2-oxobenzo[d]oxazol-3(2H)-yl)butyl)piperazin-1-yl)hexyl)benzo[d]oxazol-2(3H)-one (**5q**).* General Procedure D, yield 48%, yellow oil. ^{1}H NMR (400 MHz, D_2O) δ 7.18 (m, 8H), 3.84 (t, *J* = 6.1, 2H), 3.76 (t, *J* = 6.7, 2H), 3.53 (s, 8H), 3.21 (t, *J* = 7.3, 2H), 3.15 (t, *J* = 7.5, 2H), 1.90 – 1.54 (m, 8H), 1.31 (m, 4H). ^{13}C NMR (101 MHz, D_2O) δ 156.44, 142.36, 142.31, 130.66, 130.46, 124.41, 124.34, 123.11, 122.94, 110.18, 110.07, 109.56, 109.38, 56.78, 56.76, 56.22, 48.71, 48.66, 41.88, 41.18, 26.59, 25.17, 25.09, 24.09, 23.17, 20.73. HRMS calcd for $C_{28}H_{36}N_4O_4$ $[M+H]^+$ 492.2737, found 493.2828. Anal. calcd for $C_{28}H_{38}Cl_2N_4O_4$: C, 59.47; H, 6.77; N, 9.91. Found: C, 59.14; H, 6.53; N, 10.26.

*3-(6-(4-(4-(2-oxobenzo[d]thiazol-3(2H)-yl)butyl)piperazin-1-yl)hexyl)benzo[d]thiazol-2(3H)-one (**5r**).* General Procedure D, yield 50%, colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 – 6.82 (m, 8H), 3.98 – 3.76 (m, 4H), 2.80 – 2.06 (m, 12H), 1.75 – 1.65 (m, 4H), 1.61 – 1.24 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.73, 137.08, 137.05, 126.24, 122.93, 122.91, 122.74, 122.72, 122.60, 122.58, 110.69, 110.54, 58.49, 57.61, 53.15, 53.03, 42.69, 42.48, 27.51, 27.14, 26.66, 26.62, 25.36, 23.81. HRMS calcd for $C_{28}H_{36}N_4O_2S_2$ $[M+H]^+$ 524.2280, found 525.2384. Anal. calcd for $C_{28}H_{38}Cl_2N_4O_2S_2$: C, 56.27; H, 6.41; N, 9.37. Found: C, 56.31; H, 6.66; N, 9.01.

*3-(5-(4-(6-(2-oxobenzo[d]oxazol-3(2H)-yl)hexyl)piperazin-1-yl)pentyl)benzo[d]oxazol-2(3H)-one (**5s**).* General Procedure D, yield 55%, colorless oil. ^{1}H NMR (400 MHz, D_2O) δ 7.22 – 7.22 (m, 8H), 3.87 – 3.67 (m, 4H), 3.55 (s, 8H), 3.18 -3.14 (m, 4H), 1.81 – 1.52 (m, 8H), 1.35 – 1.30 (m, 6H). ^{13}C NMR (101 MHz, D_2O) δ 156.49, 142.39, 142.37, 130.73, 130.67, 124.41, 124.39, 123.04, 122.99, 110.16, 110.13, 109.62, 109.55, 56.74, 48.59, 48.55, 41.93, 41.67, 34.61, 26.62, 26.40, 25.20, 25.12, 23.18, 22.93, 22.76. HRMS calcd for $C_{29}H_{38}N_4O_4$ $[M+H]^+$ 506.2893, found 507.2983. Anal. calcd for $C_{29}H_{40}Cl_2N_4O_4$: C, 60.10; H, 6.96; N, 9.67. Found: C, 59.84; H, 6.81; N, 9.29.

*3-(5-(4-(6-(2-oxobenzo[d]thiazol-3(2H)-yl)hexyl)piperazin-1-yl)pentyl)benzo[d]thiazol-2(3H)-one (**5t**).* General Procedure D, yield 56%, yellow oil. ^{1}H NMR (400 MHz, DMSO) δ 7.74 – 7.08 (m, 8H), 3.96 – 3.91 (m, 4H), 3.70 – 3.06 (m, 12H), 1.91 – 1.48 (m, 8H), 1.45 – 1.18 (m, 6H). ^{13}C NMR (101 MHz, D_2O) δ 156.45, 142.31, 142.30, 130.65, 130.58, 124.33, 124.32, 122.97, 122.92, 110.08, 110.05, 109.55, 109.48, 56.76, 48.52, 48.50, 41.85, 41.59, 34.51, 34.46, 26.57, 26.35, 25.14, 25.05, 23.12, 22.87, 22.68. HRMS calcd for $C_{29}H_{38}N_4O_2S_2$ $[M+H]^+$ 538.2436, found 539.2540. Anal. calcd for $C_{29}H_{40}Cl_2N_4O_2S_2$: C, 56.94; H, 6.59; N, 9.16. Found: C, 57.15; H, 6.09; N, 9.52.

*General Procedure E. Synthesis of benzo[d]thiazole-2(3H)-thione (**6b**).* Lawesson's reagent (3.56 g, 8.82 mmol) was added, under stirring, to a solution of benzo[*d*]thiazol-2(3*H*)-one (2 g, 13.24 mmol) in anhydrous toluene (80 mL). The reaction mixture was heated under reflux for 5 h. The solvent was removed in vacuo, and the residue was purified by flash column chromatography (SiO_2) using hexane/ethyl acetate (7: 3) as eluent to give 1.4 g (63%) of **6b** as a white solid. ^{1}H NMR (400 MHz, DMSO) δ 13.72 (s, SH, 1H), 7.76 – 7.06 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 190.28, 141.70, 129.80, 127.54, 124.60, 122.14, 112.86. MS (EI) *m/z* 168 (M^++1).

*Benzo[d]oxazole-2(3H)-thione (**6a**).* General Procedure E, yield 65%, white solid. ^{1}H NMR (400 MHz, DMSO) δ 13.16 (s, SH, 1H), 7.10 – 6.67 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 187.52, 138.94, 127.04, 124.78, 121.84, 119.39, 110.10. MS (EI) *m/z* 152 (M^++1).

*General Procedure F. Synthesis of 2-(4-bromobutylthio)benzo[d]thiazole (**7b**).* K_2CO_3 (2.47 g, 17.94 mmol) and 1,4-dibromobutane (5.65 mL, 47.84 mmol) were added, under stirring, to a solution of **6b** (1.0 g, 5.98 mmol) in anhydrous DMF (20 mL). The reaction mixture was heated at 60 °C for 2 h. After cooling, the reaction mixture was poured into 70 mL water and extracted with ethyl acetate (3 x 40 mL). The combined organic layers were

washed with saturated aqueous NaCl and dried over sodium sulfate. The solvent was removed in vacuo, and the residue was purified by flash column chromatography (SiO_2) using hexane/ ethyl acetate (8: 2) as eluent to give 1.2 g (67%) of **7b** as yellow oil. ^{1}H NMR ($CDCl_3$): δ 7.95 – 7.24 (m, 4H), 3.47 (t, *J* = 5.3, 2H), 3.40 (t, *J* = 6.7, 2H), 2.16 – 1.90 (m, 4H). ^{13}C NMR ($CDCl_3$) δ 166.58, 153.23, 135.21, 126.06, 124.25, 121.51, 120.98, 32.83, 32.49, 31.52, 27.88. MS (EI) *m/z* 302 (M^++1).

*2-(4-bromobutylthio)benzo[d]oxazole (**7a**).* General Procedure F, yield 69%, dark yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 – 6.80 (m, 4H), 3.89 (t, *J* = 6.4, 2H), 3.37 (t, *J* = 5.9, 2H), 1.85 – 1.81 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.71, 136.82, 126.45, 123.14, 122.70, 122.59, 110.59, 41.66, 33.16, 29.59, 26.17. MS (EI) *m/z* 286 (M^++1).

*General Procedure G. Synthesis of 1,4-bis(4-(benzo[d]thiazol-2-ylthio)butyl)piperazine (**8b**).* K_2CO_3 (0.68 g, 4.98 mmol) and piperazine (0.07 g, 0.83 mmol) were added, under stirring, to a solution of **7b** (0.5 g, 1.66 mmol) in anhydrous DMF (10 mL). The reaction mixture was heated at 60 °C for 1.5 h. After cooling, the reaction mixture was poured into 50 mL water and extracted with ethyl acetate (3 x 30 mL). The combined organic layers were washed with saturated aqueous NaCl and dried over sodium sulfate. The solvent was removed in vacuo, and the residue was purified by flash column chromatography (SiO_2) using dichlormethane/ methanol (9.5: 0.5) as eluent to give 0.53 g (61%) of **8b** as a yellow oil. ^{1}H NMR (CDCl3): δ 7.26 (m, 8H), 3.29 (t, *J* = 7.2, 4H), 2.41 (s, 8H), 2.32 (t, *J* = 7.2, 4H), 1.82 (m, 4H), 1.62 (m, 4H). ^{13}C NMR ($CDCl_3$) δ 165.01, 151.75, 141.96, 124.18, 123.72, 118.30, 109.76, 57.80, 53.16, 32.17, 27.31, 25.81. HRMS calcd for $C_{26}H_{32}N_4S_4$ $[M+H]^+$ 528.1567, found 529.1573. Anal. calcd for $C_{26}H_{34}Cl_2N_4S_4$: C, 51.90; H, 5.70; N, 9.31. Found: C, 52.11; H, 6.17; N, 9.29.

*1,4-bis(4-(benzo[d]oxazol-2-ylthio)butyl)piperazine (**8a**).* General Procedure G, yield 63%, colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 – 7.01 (m, 8H), 3.33 (t, *J* = 7.2, 4H), 2.43 (s, 8H), 2.33 (t, *J* = 10.2 , 4H), 1.82 (m, 4H), 1.63 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.07, 153.32, 135.16, 125.98, 124.11, 121.44, 120.91, 57.86, 53.17, 33.48, 27.31, 25.94. HRMS calcd for $C_{26}H_{32}N_4O_2S_2$ $[M+H]^+$ 496.2032, found 497.2035. Anal. calcd for $C_{26}H_{34}Cl_2N_4O_2S_2$: C, 54.82; H, 6.02; N, 9.84. Found: C, 54.97; H, 6.52; N, 10.07.

4.2. Pharmacology

4.2.1. Assay for iNOS inhibition:

The assay was performed in mouse macrophages (RAW264.7, obtained from ATCC)) cultured in phenol red free RPMI medium supplemented with 10% bovine calf serum and 100 U/mL penicillin G sodium, and 100 µg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. Cells were seeded in 96-well plates (50,000 cells/well) and incubated for 24 hrs for a confluency of 75% or more. Test samples diluted in serum free medium were added and after 30 minutes of incubation LPS (5 µg/mL) was added and cells were further incubated for 24 hrs. The concentration of nitric oxide (NO) was determined by measuring the level of nitrite in the cell culture supernatant by using Griess reagent.[24] Percent inhibition of nitrite production by the test compound was calculated in comparison to the vehicle control. IC_{50} values were obtained from dose curves. L-NMMA was used as positive control.

4.2.2. Assay for NF-κB inhibition:

The assay was performed in human chondrosarcoma (SW1353, obtained from ATCC) cells as described earlier.[26] Cells were cultured in 1:1 mixture of DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin G sodium, and 100 µg/mL streptomycin at 37 °C in an atmosphere of 5% CO2 and 95% humidity. Cells (1.2×10^7) were washed once in an antibiotic and FBS-free DMEM/F12, and then resuspended in 500 µL of antibiotic-free DMEM/F12 containing 2.5% FBS. NF-κB luciferase plasmid construct was added to the cell suspension at a concentration of 50 µg/mL and incubated for 5 min at room temperature. The cells were electroporated at 160 V and one 70-ms pulse using BTX disposable cuvettes model 640 (4-mm gap) in a BTX Electro Square Porator T 820 (BTX I, San Diego, CA). After electroporation, cells were plated to the wells of 96-well plates at a density of 1.25×10^5 cells per well. After 24 h, cells were treated with different concentrations of test compound for 30 min prior to the addition of PMA (70 ng/mL) and incubated for 8 h. Luciferase activity was measured using the Luciferase Assay kit (Promega). Light output was detected on a SpectraMax plate reader. Percent inhibition of luciferase activity was calculated as compared to vehicle control and IC_{50} values were obtained from dose curves. Parthenolide was used as positive control. Sp-1 was used as a control transcription factor which is unresponsive to inflammatory mediators (such as PMA). This is useful in detecting agents that nonspecifically inhibit luciferase expression due to cytotoxicity or inhibition of luciferase enzyme activity.

4.2.3. Assay for in vitro cytotoxicity:

Cytotoxicity was determined against four human tumor cell lines [SK-MEL (malignant melanoma); KB (epidermal carcinoma, oral); BT-549 (ductal carcinoma, breast); SK-OV-3

(ovary carcinoma)] and two noncancerous kidney cell lines [Vero cells (African green monkey kidney fibroblasts) and LLC-PK11 (pig kidney epithelial cells)] as described earlier.[18] All the cell lines were obtained from ATCC and cultured in RPMI-1640 medium supplemented with bovine calf serum (10%) and amikacin (60 mg/L), at 37°C, 95% humidity, 5% CO_2. Cells were seeded at a density of 25,000 cells/well and grown for 24 h. Samples, diluted appropriately in serum free medium, were added to the cells and again incubated for 48 h. The number of viable cells was determined by Neutral Red assay.[30] Percent decrease in cell viability was calculated in comparison to vehicle control. IC_{50} (the concentration of the test compound that caused a 50% decrease in cell viability) was calculated from the dose curves. Doxorubicin was used as positive control. All assays were performed in triplicate and the mean values are given in **Table 1**.

4.2.4. In vivo assays: (Acetic acid-induced writhing in mice)

Male Swiss mice (20-30g, Harlan, Indianapolis, IN, USA) were grouped housed (n=3) in polycarbonate cages (20x35x12 cm). Food (Purina 5001 Laboratory Rodent Chow, St Louis, MO, USA) and water were available ad libitum. Room temperature was maintained at 22 +/- 1 ^{o}C and overhead illumination was maintained on a 12-h light-dark cycle.

Procedure: Acetic acid-induced writhing assay was performed as mentioned before.[31] Briefly, mice received IP injections of test compounds or vehicle 15 min before nociceptive testing. The positive control 3 mg/kg indomethacin was administered IP 30 min before nociceptive testing. Subjects received 15 min of apparatus habituation in a 15 cm diameter acrylic observation enclosure immediately before i.p. injection of 0.9% acetic acid (1 ml/ 0.1 kg). The number of writhing responses was counted for 20 minutes. In this assay, it is typical for 10 – 20% of vehicle treated animals to be non-responders.[32] In the present study 5 of the 36 subjects (i.e. 13.9%) did not respond to acetic acid injections and their data were omitted prior to statistical analyses; this yielded groups sizes of n = 4-6. Data were analyzed one-way ANOVA followed by Dunnett Multiple Comparisons Test. These procedures were conducted under the ethical standards of the International Association for the Study of Pain and in accordance with the principles of laboratory animal care as detailed in the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and were approved by the University of Mississippi IACUC (Protocol # 10-007).

4.2.5. Carrageenan-induced rat paw edema assay

Adult albino rats of both sexes weighing between 120–150 g were used. Rats were uniformly hydrated by giving 3 ml water/rat through gastric inoculation to reduce variability to edema

response. Animals were divided into groups each of five animals. The control group was given saline containing few drops of Tween 80. Test compounds or standard anti-inflammatory compound, ketorolac tromethamine, were suspended in distilled water by the aid of few drops of Tween 80 and administrated intraperitoneally one hour before induction of inflammation. Induction of inflammation was performed by S.C. injection of 50 ml of 1% carrageenan-sodium gel (Sigma–Aldrich, USA), into the subplantar region of the right hind paw. The dorso-ventral diameter (thickness) of the right and left hind paws of each rat was measured using a pair of dial thickness gauge calipers accurate to 0.0001 cm 1 h, 2 h, 3 h and 4 h after induction of inflammation. The left hind paw diameter served as a control for the degree of inflammation in the right hind paw. The percentage of anti-inflammatory activity (% inhibition of inflammation) was calculated according to the following equation:

% inhibition= $(1 - L_t/L_c) \times 100$

Lt is the mean increase in paw thickness in rats treated with the tested compounds.

Lc is the mean increase in paw thickness in control group.

Data were analyzed and results were presented in **Table 2**.

5.2. Docking study

The binding site was generated from the co-crystallized ligand (AR-C120011) within iNOS protein (PDB code: 3E65). Selected active ligands were docked using the following docking configuration: (i) number of Monte Carlo search trials = 30000, search step for torsions with polar hydrogens = 30°. (ii) The Root Mean Square Difference (RMS) threshold for ligand-to-binding site shape match was set to 2.0 employing a maximum of 1.0 binding site partitions and 1.0 site partition seed. (iii) The interaction energies were assessed employing Consistent Force Field (CFF) force field with a non-bonded cutoff distance of 10.0 Å and distance-dependent dielectric. An energy grid extending 3.0 Å from the binding site was implemented. (iv) Rigid body ligand minimization parameters were: 10 iterations of steepest descend (SD) minimization followed by 20 Broyden–Fletcher–Goldfarb–Shanno (BFGS) iterations applied to every successful orientation of the docked ligand. (v) A maximum of 10 diverse docked conformations/poses of optimal interaction energies were saved. (vi) The saved conformers/poses were further energy-minimized within the binding site for a maximum of 1000 rigid-body iterations. (vii) Six scoring functions were used to score the high ranking docked poses/conformers: Jain,[33-35] LigScore1, LigScore2,[34,35] PLP1,[36] PLP2 and PMF.[37,38]

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Scheme, Table and Figure legends:

Figure 1: Design strategy for the proposed dimers

Scheme 1: Reagents and conditions: (a) dibromoalkane, K_2CO_3, DMF, 60° C, 2 h; (b) piperazine, K_2CO_3, DMF, 60° C, 1.5 h; (c) piperazine, K_2CO_3, DMF, 60° C, 0.5 h; (d) K_2CO_3, DMF, 60° C, 2 h.

Scheme 2: Reagents and conditions: (a) Lawesson's reagent, toluene, reflux, 5 h; (b) 1,4 dibromobutane, K_2CO_3, DMF, 60° C, 2 h; (c) Piperazine, K_2CO_3, DMF, 60° C, 1.5 h.

Table 1: **Inhibition of iNOS and NF-κB activity and cytotoxicity evaluation**.

Positive controls: **L-NMMA** for iNOS inhibition, **Parthenolide** for NF-κB inhibition and **Doxorubicin** for cytotoxicity. **NA** = no activity up to 25 µg/ml. **IC_{50}** = the test concentration that caused a 50% inhibition.

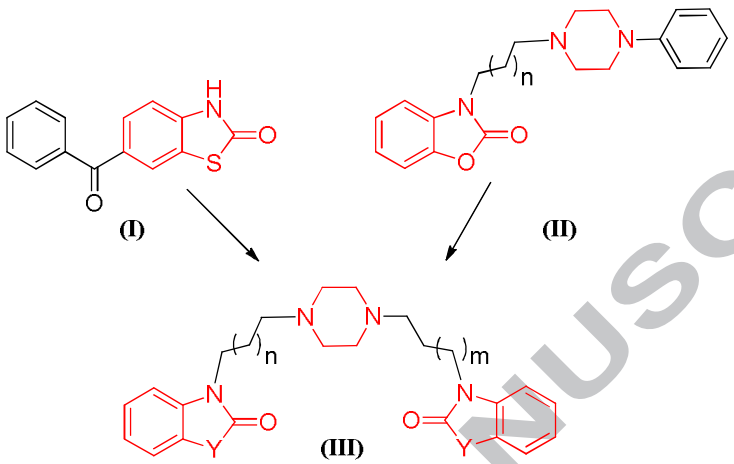
Four human cancer cell lines were used: **SK-MEL** (Melanoma), **KB** (epidermal carcinoma), **BT-549** (breast carcinoma) and **SK-OV-3** (ovarian carcinoma). Two non-cancerous kidney cell lines were also used: **Vero** (Monkey kidney fibroblast) and **LLC-PK11** (Pig kidney epithelial cells).

Figure 2: Acetic acid-induced writhing test. Writhing responses for 20 minutes were measured in mice pretreated with vehicle, Indomethacin (Indo) or test compounds after i.p. injection of 0.9% acetic acid (1 ml/ 0.1 kg). The observations are mean ± SD. * p <0.05, ** p <0.01 (n = 4-6), compared to control.

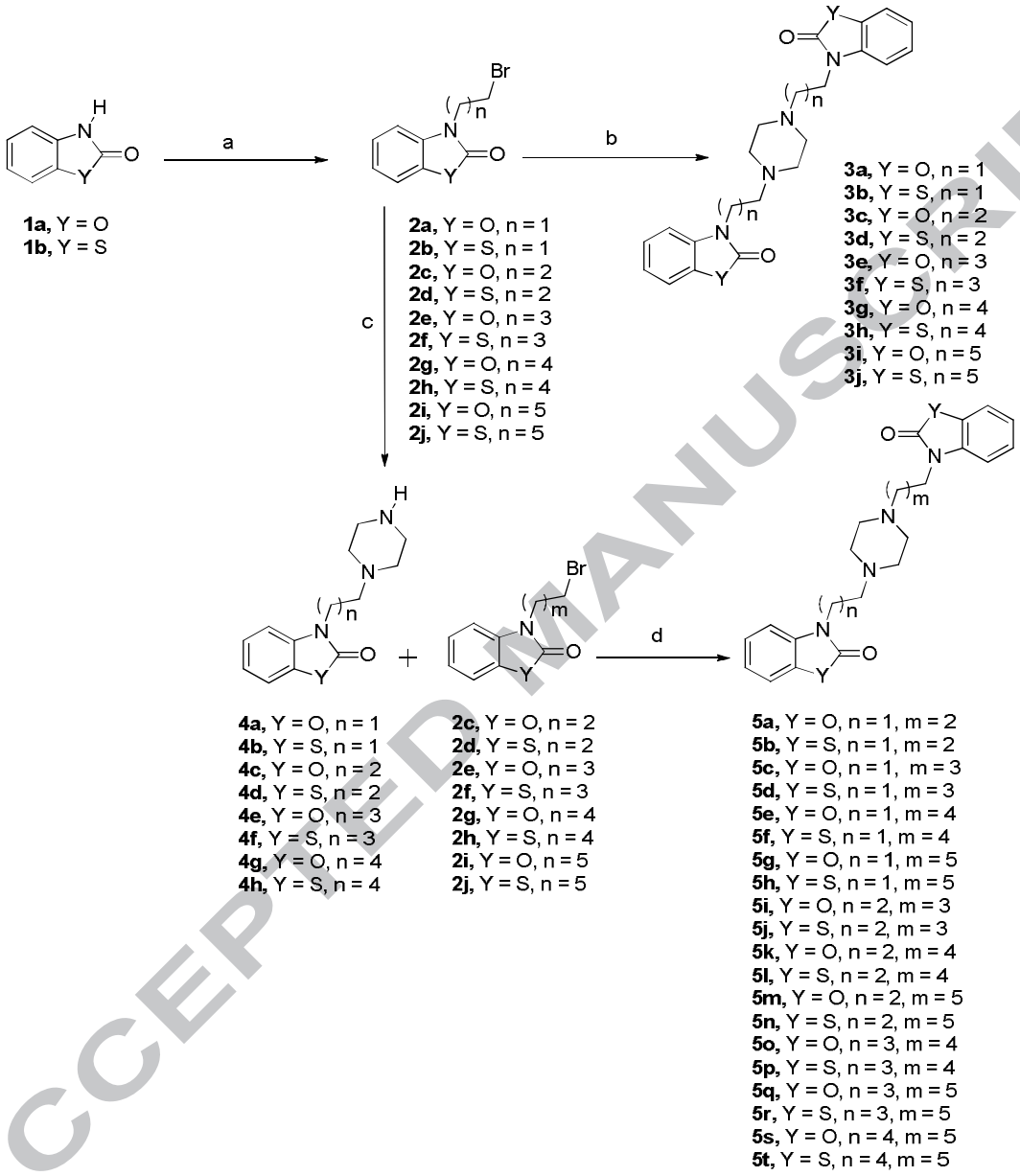
Table 2: Edema thickness and inhibition percent of control, ketorolac and tested compounds. The observations are mean ± SD. * p <0.01 (n = 5), compared to control.

Figure 3: A) Docking and binding mode of compound **8b** into iNOS active site (PDB code: **3E65), B)** Docking and binding mode of compound **5t** into iNOS active site (PDB code: **3E65),C)** Docking and binding mode of compound **3j** into iNOS active site (PDB code: **3E65),** D) The superimposition of the docked poses **3j** (blue), **5t** (pink), **8b** (green) and the crystallographic ligand, AR-C120011 (yellow) within active site of iNOS. Hydrogen bonds are represented by dashed green lines. All hydrogens are removed for the purposes of clarity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Figure 1



Scheme 1



Scheme 2

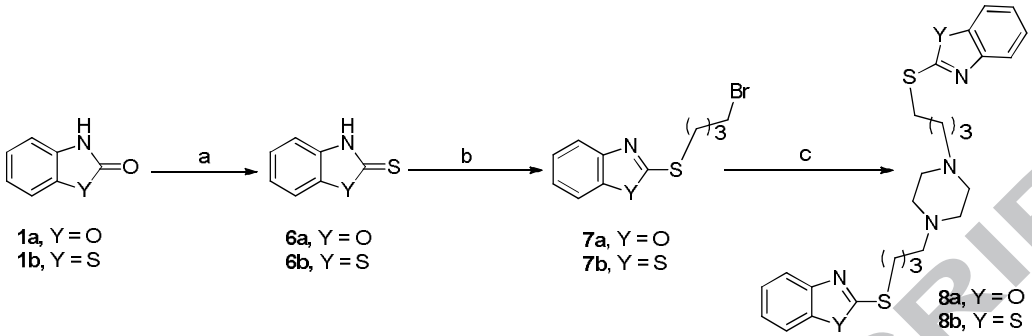


Table 1

Comp.	iNOS	NF-κB	Cytotoxicity (IC_{50} μM)					
	IC_{50} (μM)	IC_{50} (μM)	LLC-PK11	VERO	SK-OV-3	BT-549	KB	SK-MEL
3a	NA	NA	NA	NA	NA	NA	NA	NA
3b	NA	NA	NA	NA	NA	NA	NA	NA
3c	NA	4.91	NA	NA	NA	NA	NA	NA
3d	4.62	NA	23.5	NA	NA	NA	NA	NA
3e	NA	NA	NA	NA	NA	NA	NA	NA
3f	NA	2.28	NA	NA	NA	NA	NA	NA
3g	4.43	3.43	NA	19.4	NA	NA	NA	NA
3h	**0.41**	**0.43**	NA	NA	25.0	19.3	NA	19.8
3i	4.22	NA	NA	NA	NA	18.5	NA	21.3
3j	**0.28**	**0.41**	NA	NA	24.3	24.3	20.8	NA
5a	NA	NA	NA	NA	NA	NA	NA	NA
5b	NA	NA	24.1	NA	NA	NA	NA	NA
5c	NA	NA	NA	NA	NA	NA	NA	NA
5d	NA	NA	NA	NA	NA	NA	NA	NA
5e	4.98	4.78	NA	NA	NA	NA	NA	NA
5f	4.51	1.71	NA	23.8	NA	NA	NA	NA
5g	4.66	3.07	NA	NA	NA	NA	NA	NA
5h	3.43	2.95	NA	NA	NA	25	NA	25
5i	NA	NA	NA	NA	NA	NA	NA	NA
5j	4.51	1.71	19.2	NA	NA	NA	NA	NA
5k	4.86	NA	NA	NA	NA	NA	NA	NA
5l	**2.55**	**1.46**	NA	NA	NA	14	NA	16.8
5m	4.50	NA	NA	NA	NA	NA	NA	NA
5n	2.57	2.40	NA	NA	NA	25	NA	12
5o	4.54	NA	NA	17.6	NA	NA	NA	NA
5p	**1.44**	**1.18**	NA	NA	NA	22.1	NA	17.5
5q	**1.77**	**1.24**	NA	NA	NA	NA	NA	NA
5r	**0.51**	**0.83**	NA	NA	NA	21.3	19.5	14.5
5s	**2.90**	**1.40**	NA	NA	NA	22.5	NA	23
5t	**0.29**	**1.06**	NA	25	25	19.6	NA	NA
8a	**1.12**	**1.40**	23.5	NA	NA	22.5	25	25
8b	**0.41**	**0.34**	NA	NA	NA	NA	25	17.5
L-NMMA	**2.30**	-	-	-	-	-	-	-
Parthenolide	-	**2.57**	-	-	-	-	-	-
Doxorubicin	-	-	**0.79**	**0.92**	**0.20**	**0.17**	**0.22**	**0.14**

Figure 2

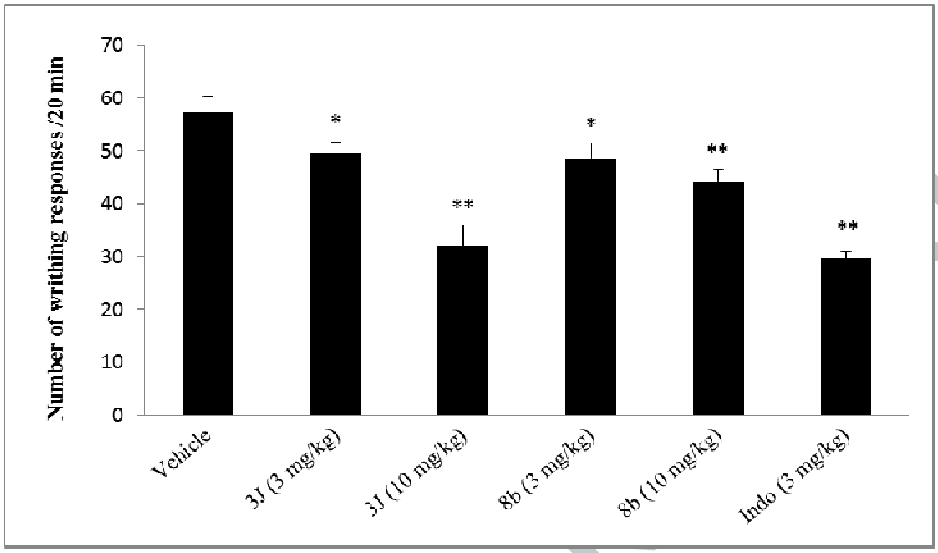


Table 2

Compound	Edema thickness (x10^{-2} mm) ±SEM (Inhibition %)			
	1h	2h	3h	4h
Control	140.8 ± 1.35	155.6 ± 2.80	167.4 ± 2.09	195.6 ± 3.02
Ketorolac 30 mg/kg	41.6 ± 2.02* (70.46)	40.2 ± 2.82* (74.16)	41.2 ± 2.06* (75.38)	40.4 ± 1.52* (79.34)
3j 30 mg/kg	95.50 ± 1.02* (32.17)	97.24 ± 2.35* (37.51)	101.6 ± 1.68* (39.31)	123.5 ± 1.13* (36.86)
5t 30 mg/kg	93.01 ± 1.93* (33.94)	91.57 ± 2.63* (41.15)	109.24 ± 1.36* (34.74)	127.34 ± 1.87* (34.89)
8b 30 mg/kg	56.48 ± 2.14* (59.89)	55.6 ± 2.09* (64.27)	61.32 ± 1.67* (63.37)	103.54 ± 1.77* (47.07)

Figure 3