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Title Page

Divergent Cytotoxic and Metabolically Stimulative Functions of Sigma-2 Receptors: Structure-activity Relationships of 6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one (SN79) Derivatives

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Running Title Page

Divergent cytotoxic and metabolic sigma-2 receptor effects

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ABBREVIATIONS: CM572, 3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-6-isothiocyanatobenzo[*d*]oxazol-2(3*H*)-one; CM764, 6-acetyl-3-(4-(4-(2-amino-4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one; DTG, 1,3-di-o-tolylguanidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIF1α, hypoxia-inducible factor 1 alpha; MEM, Minimal Essential Media; MTT, 3-[4,5 dimethylthaizol-2-y]-2,5 diphenyltetrazolium bromide; -NCS, isothiocyanate substituent; PGRMC1, progesterone receptor membrane component 1; PhNCS, phenyl isothiocyanate; ROS, reactive oxygen species; RT, room temperature; SN79, 6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one; Tris, tris(hydroxymethyl)aminomethane; VEGF, vascular endothelial growth factor.

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Abstract

Sigma-2 receptors, recently identified as TMEM97, have been implicated in cancer and neurodegenerative disease. Structurally distinct sigma-2 receptor ligands induce cell death in tumor cells, linking sigma-2 receptors to apoptotic pathways. Recently, we reported that sigma-2 receptors can also stimulate glycolytic hallmarks; effects consistent with a pro-survival function and upregulation in cancer cells. Both apoptotic and metabolically stimulative effects were observed with compounds related to the canonical sigma-2 antagonist, SN79. Here we investigate a series of 6-substituted SN79 analogs in order to assess the structural determinants governing these divergent effects. Substitutions on the benzoxazolone ring of the core SN79 structure resulted in high-affinity sigma-2 receptor ligands (K_i = 0.56–17.9 nM), with replacement of the heterocyclic oxygen by –NMe (N-methylbenzimidazolones) generally decreasing sigma-1 affinity and a sulfur substitution (benzothiazolones) imparting high affinity at both subtypes, lowering subtype selectivity. Substitution at the 6-position with -COCH₃, –NO₂, -NH₂, or –F resulted in ligands that were not cytotoxic. Five of these ligands induced an increase in metabolic activity, as measured by increased reduction of MTT in human SK-N-SH neuroblastoma cells, further supporting a role for sigma-2 receptors in metabolism. Substitution with 6-isothiocyanate resulted in ligands that were sigma-2 selective and that irreversibly bound to the sigma-2 receptor, but not to sigma-1. These ligands induced cell death upon both acute and continuous treatment (EC₅₀ = 7.6 - 32.8 &[mu]M), suggesting that irreversible receptor binding plays a role in cytotoxicity. These ligands will be useful for further study of these divergent roles of sigma-2 receptors.

Introduction

The sigma-2 receptor is a 21.5 kDa protein, recently identified as the known protein TMEM97 (Hellewell and Bowen, 1990; Hellewell et al., 1994; Alon et al., 2017). Due to its upregulation in tumors compared to healthy tissue, the sigma-2 receptor (sigma-2R/TMEM97) has been implicated in cancer cell proliferation as well as developed into a tool for tumor imaging (Vilner et al., 1995; Mach et al., 1997; Wheeler et al., 2000; Mach and Wheeler, 2009; Zeng et al., 2011; Shoghi et al., 2013). Additionally, a connection between sigma-2 receptors and Alzheimer's disease has been described (Izzo et al., 2014a,b; Sahlholm et al., 2015; Yi et al., 2017). The sigma-2 receptor may also have some relationship to progesterone receptor membrane component 1 (PGRMC1), though the two proteins are not synonymous as initially proposed (Xu et al., 2011; Abate et al., 2015; Chu et al., 2015). They are both upregulated in cancer, both appear to be involved in regulation of cell viability, are largely co-localized in cells, and isolation of PGRMC1 was achieved using a sigma-2 selective probe. Thus, it is imperative that the pharmacology of the sigma-2 receptor be further developed and described in order to understand the pathways, mechanisms, and functions associated with it.

Functional classification of sigma-2 receptors is not standardized, with different investigators preferring different criteria for characterization. Generally, agonists of the sigma-2 receptor are considered to induce programmed cell death. Cleavage of Bid, caspase-3 activation, induction of an immediate rise in cytosolic calcium, and positive TUNEL staining have been proposed as markers of sigma-2 receptor agonist activity (Vilner and Bowen, 2000; Crawford and Bowen, 2002; Hazelwood and Bowen, 2006; Wang and Bowen, 2006; Zeng et al., 2014; Nicholson et al., 2015). Recently, we described a novel function of sigma-2 receptors, where a sigma-2 receptor ligand, CM764, induced an increase in various markers of glycolytic cellular

metabolism as initially assessed by an increase in MTT reduction, without inducing changes in cell viability or cell proliferation (Nicholson et al., 2016). This effect was blocked by various sigma-2 receptor antagonists. In addition to the increase in MTT reduction, the effect comprised an increased level of NAD+/NADH and ATP, a marked decrease in basal ROS level, and an increase in HIF1α which may be connected to an observed increase in VEGF expression (Nicholson et al., 2016). Thus sigma-2 receptors may have a dual role with respect to cellular survival and may activate different pathways depending on the ligand. How this occurs is not clear. Interestingly, some of the effects of CM764 are similar to those mediated by PGRMC1, which has previously been associated with the sigma-2 receptor, as mentioned above (Xu et al., 2011; Abate et al., 2015; Chu et al., 2015). Notably, PGRMC1 has been implicated in stimulation of VEGF gene expression (Neubauer et al., 2009). PGRMC1 generally has pro-proliferative, pro-survival, and anti-apoptotic effects in cells (Losel et al., 2008; Neubauer et al., 2009; Ahmed et al., 2010; Peluso et al., 2010). It is conceivable that PGRMC1 is involved in sigma-2/TMEM97 signaling in some way or that some sigma-2 ligands interact directly with PGRMC1. However, more studies would be necessary to address this.

A significant challenge for study of the sigma-2 receptors is the paucity of highly sigma-2 selective ligands. There exist several sigma-1-selective ligands, but ligands with high selectivity for sigma-2 over sigma-1 are relatively scarce. SN79 is a moderately selective sigma-2 receptor antagonist (Ki = 7 and 28 nM at sigma-2 and sigma-1, respectively) (Kaushal et al., 2011). Here, we characterize a novel series of SN79 analogs with single-element variations at the 6-position of the benzoxazolone, N-methylbenzimidazolone, and benzothiazolone heterocyclic systems. The study sought to determine a structural foundation for sigma-2 receptor affinity and selectivity. Furthermore, both cytotoxic effects and metabolic stimulative effects were induced

by compounds within the same SN79-related structural class in various cell lines. CM572 induced programmed cell death (Nicholson et al., 2015), while CM764 induced metabolic stimulation (Nicholson et al., 2016). Thus we further explored structural determinants of these divergent effects within this class. The study provides further evidence supporting a dual role of the sigma-2 receptor and provides a foundation from which to design targeted sigma-2 receptor ligands that are highly selective and result in inclusion into a predicted functional class.

Materials and Methods

2.1 Compound Syntheses

The synthesis and characterization of compounds are described in the supplemental information to this manuscript (Supplemental Methods).

2.2 Radioligand Binding Assays

Radioligand competition binding assays were performed as previously described (Nicholson et al., 2015). Briefly, 150 µg rat liver membrane protein was incubated for 120 minutes at 25ºC in 20 mM HEPES, pH 7.4 with varying concentrations of each novel ligand and 3 nM [^{3}H](+)-pentazocine (PerkinElmer, Waltham, MA) for measuring sigma-1 receptors or 5 nM [^{3}H]DTG (PerkinElmer, Waltham, MA) to measure sigma-2 receptors in the presence of 100 nM unlabeled (+)-pentazocine to mask sigma-1 receptors in a final volume of 0.5 mL. Haloperidol (10 µM) was used to determine nonspecific binding. After incubation, assays were terminated by dilution with 5 mL ice cold 10 mM Tris pH 7.4 and filtration through 0.5% polyethyleneimine-soaked fiberglass filters using a Brandel Cell Harvester (Brandel, Gaithersburg, MD) and two additional 5 mL washes in the same buffer. GraphPad Prism 6 software (GraphPad Software, La Jolla, CA) was used to determine Ki values with [^{3}H](+)-pentazocine K_d=7.5 nM at sigma-1 receptors in rat liver homogenates and [^{3}H]DTG K_d=17.9 nM at sigma-2 receptors in rat liver homogenates.

For irreversible binding studies, 0.3 mg protein/mL membrane homogenate was treated with the indicated concentration of each isothiocyanate-substituted ligand individually for 60 minutes in 20 mM HEPES pH 7.4 at 25ºC. This preparation was then diluted to 0.018 mg protein/mL using ice-cold 20 mM HEPES and centrifuged for 10 min at 37,000 x g. The pellet was resuspended to the original volume with ice-cold buffer and centrifuged again, and then resuspended again to the original volume. Non-covalently bound ligand was then allowed to dissociate during a 60 minute incubation at 25ºC prior to centrifugation of the preparation at 37,000 x g for 10 minutes and resuspension of the pellet in 50 mM Tris, pH 8.0 to a concentration of 0.6 mg/mL. This homogenate was used directly in binding studies at a final protein concentration of 0.3 mg protein/ml in 0.5 mL total volume in incubation with [^{3}H](+)-pentazocine and [^{3}H]DTG (with unlabeled (+)-pentazocine) to determine recovery of sigma-1 and sigma-2 binding sites, respectively. Control membranes were treated in the same manner without exposure to ligand. This membrane preparation procedure was able to dissociate 500 nM SN79 from both sigma-1 and sigma-2 receptors, indicating successful removal of non-irreversibly bound ligand.

2.3 Cell Culture

Human SK-N-SH neuroblastoma cells (ATCC, Manassas, VA) were cultured in MEM (Gibco, Grand Island, NY) containing 10% fetal bovine serum, 10 mg/L human insulin, and 1X Pen-Strep (Gibco, Grand Island, NY) in a humidified atmosphere at 37 ºC and 5% CO_2. Cells were passaged at 70% confluency.

2.4 Cell Viability and Metabolic Stimulation Assays

Cell viability was measured using MTT assays (Trevigen, Gaithersburg, MD). Cells were plated in 96-well plates at 15,000 cells/well and allowed to attach overnight. Cells were then treated with the indicated concentrations of ligand in a final volume of 100 µL for 24 hours. After this period, 10 µL of MTT Reagent was added to each well and allowed to be metabolized for 3 hours at 37 ºC, then 100 µL MTT Detergent Reagent was added and allowed to solubilize formazan crystals and cell membranes for 2 additional hours. Absorbance was then read at 570 nm. A decrease in absorbance was indicative of a loss of viable cells, and percent cytotoxicity was calculated using the percent loss in formazan formation in treated cells as compared to untreated cells by the following formula:

% Cytotoxicity = 100 – [(O.D. treated cells – O.D. media blank / O.D. untreated control cells – O.D. media blank) x 100]

EC_{50} values for dose-response curves were determined using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA).

For acute treatments, cells were treated with ligand for 60 minutes and then washed twice with fresh ligand-free media to remove any non-irreversibly bound ligand. Fresh ligand-free media (100 µL) was then added to each well and allowed to incubate for 24 hours at 37 ºC prior to MTT assay as described above.

Some compounds in this study were found to produce an increase in MTT reduction relative to control, as opposed to a decrease, in the 24 h period of incubation. This is indicative

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of a stimulation in cellular metabolism. Metabolic stimulation was represented as percent change in MTT reduction and was calculated using the following formula:

% Change in MTT Reduction = [100 x (O.D. treated cells – O.D. media blank) / (O.D. untreated control cells – O.D. media blank)] – 100

Results

3.1 Binding affinity at sigma-1 and sigma-2 receptors

SN79 was used as the lead compound in this study. SN79 (Table 1) has a benzoxazolone heterocycle, where X=O. Analogous benzothiazolones (X=S) and N-methylbenzimidazolones (X=NMe) were synthesized. Within the three heterocyclic families, various substitutions at the 6-position were examined, including compounds were R= acetyl (-$COCH_3$), isothiocyanate (-NCS), nitro (-NO_2), and amine (-NH_2). All ligands were examined for their ability to bind sigma-1 and sigma-2 receptors using [^{3}H](+)-pentazocine to measure sigma-1 receptors and [^{3}H]DTG to measure sigma-2 receptors in the presence of unlabeled (+)-pentazocine to mask sigma-1 receptors as described in Materials and Methods. Results are summarized in Table 1.

When ligands with the same R-group were compared, there existed a trend towards a loss of high-affinity binding at sigma-1 receptors for compounds with –NMe- X-group substitutions. This was most clearly exemplified with WA403 (R=NH_2) (and to a lesser extent with NF7 (R=$COCH_3$)), where there is a striking rightward shift in the sigma-1 binding competition curves for the –NMe- substituted ligand when compared to the other ligands with R=NH_2 and either X=O or X=S, as demonstrated in Figure 1. When X=NMe, the sigma-1 affinity was decreased by at least 6.5-fold for R=$COCH_3$ in the same comparisons. When R=NO_2, the X=NMe substitution still demonstrated the lowest affinity binding as compared to other X-group substitutions, however the shift was less drastic. For WA404 (R=NCS) the –NMe- substituted ligand bound sigma-1 receptors with ~8 times lower affinity than the corresponding X=S substitution (WA435). For CM572 (R=NCS, X=O), a valid Ki value could not be determined at sigma-1 receptors due to solubility limits of the ligand. However, it is clear that CM572 (X=O,

R=NCS) is an outlier in this series due to its lack of significant sigma-1 receptor binding and therefore its extreme selectivity.

Comparing ligands with the same X-group substitution allowed for a comparison of the effects of R-group substitutions on sigma-1 receptor binding affinity. Ligands with R=NCS generally had a significantly decreased affinity for sigma-1 receptors as compared to any other R-group substitution. This is best demonstrated by CM572 (R=NCS, X=O), where the isothiocyanate substituted ligand has a decrease in sigma-1 receptor binding affinity of over 500-fold when compared to any other R-group substitution, as shown in Figure 2. For X=S substitutions, there was at least a 3-fold loss of sigma-1 affinity for the isothiocyanate (WA435) substitution as compared to the other R-group substitutions. As mentioned above, sigma-1 receptor binding affinity was not high for any ligand when X=NMe (Figure 1), and WA404 (R=NCS, X=NMe) had a low affinity Ki value of 449 nM at the sigma-1 receptor, although this was not the lowest affinity of the X=NMe substituted ligands.

It is not immediately clear why, within each heterocyclic system, the R=NCS analogs would demonstrate lower affinity for sigma-1 compared to the other substituents. They do not covalently bind to the sigma-1 receptor (see below), presumably due to the lack of a properly oriented receptor nucleophile in or near the binding site. However, this would not necessarily affect sigma-1 binding affinity. It is possible that there are steric issues affecting fit of the isothiocyanate group into the binding pocket. Another possibility is that these ligands may assume a "flipped" binding conformation in the sigma-1 binding pocket. Electronic effects may also come into play, since sigma-1 affinity of the isothiocyanates is affected by the nature of the -X-moiety in the heterocycle. Further studies to determine ligand bound X-ray crystal structures would be needed to confirm these possibilities.

The effect of X-group substitutions on receptor affinity was not limited to the sigma-1 subtype. When sigma-2 affinities were compared, X=S substituted ligands had generally high sigma-2 affinity as compared to X=O and X=NMe substituted ligands. Ligands with varying X group substitutions and either R=NCS (left panel) or R=NH_2 (right panel) are shown in Figure 3 for sigma-2 receptor binding. WA435 (R=NCS, the X=S) showed at least a 7-fold increase in sigma-2 receptor affinity as compared to other X-group substitutions (left panel). Similarly, when R=NH2, the sulfur X group substitution resulted in higher affinity ligands compared to X=O and X=NMe substitutions (right panel). Similar results were also observed when R=$COCH_3$. Since X=S substitution resulted in ligands with sigma-2 receptor affinities that were between 2-4 nM, this indicates that the benzothiazolone heterocycle represents an optimal platform for further development of high affinity sigma-2 ligands. Furthermore, the lower sigma-1 affinity of the R=NCS derivative resulted in WA435 having 28.5-fold selectivity for sigma-2 receptors.

Across all three heterocycles, R=NO_2 substitutions generally resulted in very high affinity sigma-2 ligands, with Ki values better than 8 nM. Of particular note is CM458 (X=O, R=NO_2) which exhibited subnanomolar affinity at sigma-2 receptors (Ki = 0.56 nM).

The variations in the X-position that resulted in drastic effects on binding affinity at both sigma-1 and, to a lesser extent, sigma-2 receptor binding affinity naturally also demonstrated an effect on subtype selectivity of the ligands. It should be noted that all ligands demonstrated at least slightly selective binding for the sigma-2 receptor over the sigma-1 receptor except for CM571 (X=O, R=NH_2). Generally, having an oxygen in the X-position led to more highly sigma-2 selective ligands, while having a sulfur in this position generally decreased selectivity

(Table 1). However, this trend was less closely adhered to than trends in binding at each individual receptor.

Overall the modifications yielded five new compounds that have higher sigma-2 receptor binding affinity than the parent compound, with CM458 exhibiting 12.3-fold higher affinity than SN79. They also resulted in seven ligands that are significantly more selective for sigma-2 relative to sigma-1, compared to SN79. Notably, WA435, CM458, WA403, and CM572 were respectively, 28.5-, 39.6-, 167-, and >685-fold selective for sigma-2 over sigma-1. This is a marked improvement over SN79 which was only 4-fold selective.

3.2 Irreversible binding capability at sigma-1 and sigma-2 receptors

The isothiocyanate moiety was added with the idea of imparting irreversible binding capability to the ligands through potential nucleophilic attack from an amine or thiol group appropriately positioned in the binding pocket of the sigma-2 receptor. We have previously shown that CM572 (X=O, R=NCS) is able to irreversibly bind to the sigma-2 receptor (Nicholson et al. 2015). In the current study, we examined two additional R=NCS substituted ligands for their ability to irreversibly bind sigma-1 and sigma-2 receptors. Figure 4 shows results of ligand washout experiments. Both WA404 (X=NMe, R=NCS) and WA435 (X=S, R=NCS) were able to irreversibly bind sigma-2 receptors, with WA435 (X=S, R=NCS) having a higher potency for irreversible binding (Figure 4). This trend correlates with sigma-2 receptor binding affinity differences, as the X=S substituted ligands have a higher affinity for sigma-2 receptors than the X=NMe substituted ligands. Neither isothiocyanate potently irreversibly bound sigma-1 receptors, although WA404 (X=NMe, R=NCS) did show some ability to

irreversibly block sigma-1 receptor binding at high doses. Interestingly, despite the relatively higher affinity of WA435 for sigma-1 receptors and saturation at the highest concentration examined (1,000 nM), the compound was completely washed out of the receptor, confirming lack of a suitable neighboring nucleophile in sigma-1 receptor.

3.3 Effect of acute and continuous exposure to ligand on cell viability in SK-N-SH neuroblastoma

One generally accepted criterion for classification of a sigma-2 receptor ligand as an agonist is the ability to induce cell death. In order to classify the function of the ligands in this study, MTT cell viability assays were used to determine the efficacy and potency of the ability of each ligand to induce cell death in SK-N-SH neuroblastoma. The R-group substitution had the greatest effect on the ability of each ligand to induce cell death. The only compounds in the series with the ability to potently decrease cell viability were those with R=NCS, regardless of X-group substitution. Data for the most active compounds is depicted in Figure 5. The isothiocyanate substituted ligands all were able to induce a reduction in cell viability in the SK-N-SH neuroblastoma with an EC_{50} below 35 μM, with CM572 (X=O, R=NCS) being the most potent with an EC_{50} of 7.6 μM, as previously reported (Nicholson et al., 2015). Isothiocyanates with other X-group substitutions (X=NMe and X=S) demonstrated less potent EC_{50} values (WA404=30.4 ± 1.1 μM and WA435=32.8 ± 1.1 μM). Some non-isothiocyanate ligands were also able to induce cell death, but were much less potent than the isothiocyanates. WA413 (X=S, R=NO_2) reached ~36% cytotoxicity at a dose of 50 μM after 24 h treatment. An EC_{50} value for

this ligand could not confidently be determined due to insolubility at high doses. WA402 (X=NMe, R=NO_2) exhibited <50% cell death at a concentration of 100 μM.

The radioligand binding study demonstrated that the isothiocyanate substitution did impart the ability of the ligands to irreversibly bind sigma-2 receptors in each heterocycle class, while sigma-1 receptor irreversible binding was not generally observed (Figure 4). The effect of this irreversible binding on cell viability was further tested by treating SK-N-SH neuroblastoma with an acute 60 minute pretreatment with the isothiocyanate ligands, followed by extensive washing and a 24 hour incubation in fresh ligand-free media. Cell viability was assessed after this period, and any induced cell death was attributed to ligand irreversibly bound and therefore continuously activating sigma-2 receptors throughout the 24 hour incubation without free ligand. Results are shown in Figure 6. All isothiocyanate derivatives were found to continue to induce cell death after the acute 60 minute pretreatment, washing, and 24 hour incubation in media without free ligand. For both CM572 (X=O, R=NCS) and WA404 (X=NMe, R=NCS), the acute treatment reached comparable levels of cell death as the 24 hour incubation with ligand, with no statistically significant differences between the two exposure times ($p=0.13$ and $p=0.056$ for comparison of 24 hour incubation with ligand and 60 minute acute treatment for CM572 and WA404, respectively, Student's t-test). Interestingly, for WA435 (X=S, R=NCS) the efficacy of the ligand increased when treated acutely as compared to the 24 hour continuous exposure ($p=0.003$ for comparison of the two conditions, Student's t-test). It should be noted here that in our previous study using siramesine, removing siramesine (which cannot bind irreversibly to sigma receptors) after a 60 min pretreatment of SK-N-SH neuroblastoma cells completely prevented induction of cell death (Nicholson et al. 2016). This rules out the possibility that a 60

min acute exposure to sigma-2 agonist is enough time to commit to a cell death program, such that cell death would occur after subsequent removal of even a reversibly bound ligand.

When cell viability data from all the compounds is considered, the data clearly shows that in order for the compounds in this SN79-related series to be potently cytotoxic, the isothiocyanate group must be present. This could indicate that irreversible binding to sigma-2 receptors is a requirement for cytotoxicity. However, another explanation for the efficacy of the isothiocyanate ligands is that the strong electron-withdrawing character of this moiety influences the receptor interaction such that the efficacy is altered. In order to discriminate between the effect of irreversible binding and the effect of the strong electron withdrawing character of the isothiocyanate moiety on cell viability, an R-group substitution with fluorine was investigated to represent the strong electron withdrawing character of the isothiocyanate but without the capability to irreversibly bind to the sigma-2 receptor. This ligand, WA379 (X=NMe, R=F), bound with high affinity and selectivity to sigma-2 receptors (Table 1; Ki=100.7 nM at sigma-1 receptors and 6.1 nM at sigma-2 receptors). When the effect of treatment with the fluorine substituted ligand on cell viability was examined, no significant cytotoxicity was induced (data not shown). Instead, a metabolic stimulation effect was observed (see below). These results demonstrate that the strong electron withdrawing character of the isothiocyanate group alone is not sufficient to induce significant levels of cell death, while irreversible binding was sufficient to induce cytotoxicity.

Another possible explanation for the observation that the isothiocyanate derivatives are the most potent cytotoxic ligands is that the effect is due to a non-specific off target effect of the isothiocyanate and that the sigma-2 receptor is playing no role. The –NCS moiety could be attacked by a nucleophile on any protein which could result in general toxicity to the cell,

especially in the higher micromolar range. We examined this by assessing the effect of phenylisothiocyanate (PhNCS) on sigma receptor binding and SK-N-SH cell viability. PhNCS would not be expected to exhibit sigma receptor affinity, but should readily enter cells and potentially be attacked by protein nucleophiles. Figure 7 (panel A) shows that PhNCS indeed had no appreciable effect on sigma-1 or sigma-2 receptor binding at concentration up to 100 μM. Figure 7 (panel B) shows the effect of PhNCS on cell viability in media with two concentrations of fetal bovine serum, 2% and the normal 10%. High and low concentrations of FBS were used to examine possible serum effects on the –NCS group, though concentration of FBS was found to have no effect on activity of CM572 (X=O, R=NCS) in our previous study (Nicholson et al. 2015). There was no significant cytotoxic effect of PhNCS on SK-N-SH cells. A dose of 10 μM induced no significant cytotoxic effect, and there was < 25% cytotoxicity at 100 μM PhNCS over a 24 h incubation period at either FBS concentration. Taken together, the data show that specifically targeting the isothiocyanate to the sigma-2 receptor is necessary to affect both binding activity and cytotoxic efficacy of the isothiocyanate SN79 derivatives. Thus, it appears that irreversible sigma-2 receptor binding activity imparted by the isothiocyanate moiety may play a key role in the cell death-inducing activity of these analogs.

3.4 Effect of ligand treatment on metabolic reduction of MTT

We have recently described a novel, non-toxic, non-zero function for sigma-2 receptors using the SN79 derivative CM764 (Nicholson et al., 2016). Five of the ligands investigated in this series replicated this effect, inducing an increase in reduction of MTT upon 24 h treatment as compared to untreated cells. Results are shown in Figure 8. The five ligands (CM458, CM571,

NF7, WA504, and WA379) that promoted significantly increased MTT reduction in SK-N-SH neuroblastoma all had high sigma-2 receptor binding affinity, with receptor subtype selectivity varying widely. These compounds have R-group substitutions representing all possibilities examined except R=NCS, which was shown to be toxic in all cases, as described above. Additionally, this subset of five ligands encompasses all three possible X-group substitutions examined. These data indicate that neither X-group nor R-group alone determine the ability to induce increased reduction of MTT.

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Discussion

The goal of this study was to establish a relationship between ligand structure, binding affinity and selectivity, and functional efficacy of a series of novel SN79 derivatives at sigma-1 and sigma-2 receptors. The parent compound, SN79 displays ~4-fold selectivity for the sigma-2 receptor over sigma-1, and has been screened for off-target binding without any significant hits (Kaushal et al., 2011). SN79 is a sigma-2 receptor antagonist, and does not induce cell death even at high doses (Garcia, 2012). As one of the few sigma-2 selective ligands that has been thoroughly characterized, this was a natural starting point for development of additional sigma-2 receptor selective ligands with which to study structural parameters affecting receptor affinity and ability to induce either cell death or a metabolically stimulative effect in SK-N-SH neuroblastoma cells. We synthesized a series of ligands with single-moiety changes using the core structure of SN79 to examine these relationships.

Table 1 shows binding affinity of the analogs at sigma-1 and sigma-2 receptors. Affinity at sigma-1 receptors was significantly decreased for ligands with X=NMe substitutions, suggesting potential steric strain at this position in the binding pocket of the sigma-1 receptor (Figure 1). Sterically smaller X-group substitutions (X=O and X=S) did not result in a general decrease in sigma-1 receptor binding affinity, lending strength to this hypothesis. Another possibility is that the sigma-1 receptor binding pocket may have an ideally situated hydrogen bond donor that the X=O and, to a weaker extent, the X=S substitutions could participate in to promote higher affinity binding. However, this is a less likely cause as X=S substitutions had generally higher affinity binding, yet sulfur is a much weaker hydrogen bond acceptor than oxygen. Another factor that demonstrated a decrease in sigma-1 receptor binding affinity is the isothiocyanate R-group substitution. Derivatives with R=NCS generally had lower affinity for

the sigma-1 receptor compared to the other substitutions, which resulted in a trend of increased selectivity for the sigma-2 receptor. All ligands in this series except CM571 (X=O, R=NH_2) demonstrated at least slight selectivity for the sigma-2 receptor, which was maintained from the parent compound SN79.

Sigma-2 receptor binding affinity was increased for ligands with R=NO_2 substitutions (Figure 2) and independently for ligands with X=S substitutions (Figure 3). The X=S substitution also generally resulted in an increase in affinity at sigma-1 receptors (although to a lesser extent), which resulted in reduced sigma-2 receptor selectivity. However, compared to sigma-1 affinity, sigma-2 receptor affinity appears to be less dependent on interplay between X- and R-group substituents, with compounds generally having high affinity at sigma-2 receptors. These results are consistent with the ligand core structure, in which the X-position and R-position are conjugated. Therefore, resonance allows for changes in the X-group to affect electron density at the R-position, and vice versa.

Substitution of the R-group with an isothiocyanate allowed for the possibility of irreversible binding if there were an ideally positioned amine (lysine) or thiol (cysteine) group in the binding pocket of the receptor. This irreversible binding capability was confirmed by measuring recovered radioligand binding after pretreatment with isothiocyanate-substituted ligands followed by washout of any non-irreversibly bound ligand. All isothiocyanate substituted ligands were able to irreversibly bind sigma-2 receptors, while only WA404 (X=NMe, R=NCS) showed any ability to irreversibly bind sigma-1 receptors and not with high potency. This indicates that the isothiocyanate in the R-position may be ideally placed for covalent bond formation in the sigma-2 receptor binding pocket, but not in the sigma-1 receptor binding pocket.

Interestingly, only R=NCS substituted ligands in this series were able to induce significantly potent levels of cell death in SK-N-SH neuroblastoma, indicating agonist activity (Figure 5). This ability to induce cell death appears to be a result of the capacity to irreversibly bind sigma-2 receptors, rather than a result of the highly electron withdrawing group in the R-group position, as neither R=NO_2 substituted ligands nor an R=F substituted ligand were able to induce significant cell death in comparable concentration range. These data indicate that irreversible binding is sufficient to induce cytotoxicity, while simply having a highly electron withdrawing group in the same position is not. Furthermore, cytotoxicity of the isothiocyanate required specific targeting to the sigma-2 receptor, since phenyl isothiocyantate, which lacks sigma receptor affinity, had little effect on cell viability. Interestingly, while irreversible binding may play some role with SN79 analogs, irreversible binding is clearly not required for sigma-2 receptor-induced cell death in compounds from other structural classes, such as CB-64D, siramesine, and SV119, that lack an –NCS group and the ability to bind covalently.

We surmised that irreversible sigma-2 binding would impart long-lasting effects on sigma-2 receptor function. When SK-N-SH cells were acutely treated with isothiocyanate substituted ligands for 60 minutes followed by extensive washing and 24 hour incubation in media without free ligand, the levels of cytotoxicity achieved were comparable to those after 24 hour continuous incubation with ligand for both CM572 (X=O, R=NCS) and WA404 (X=NMe, R=NCS) (Figure 6). This indicates that the ability to irreversibly bind sigma-2 receptors also imparts the ability to continue to activate these receptors and induce cytotoxicity without free ligand present in the media. WA435 (X=S, R=NCS) was found to induce cell death more potently when acutely treated and then incubated in media without free ligand for 24 hours as compared to a 24 hour continuous exposure to ligand. WA435 is the only isothiocyanate ligand

that retains significant affinity for the sigma-1 receptor, although it does not irreversibly bind the sigma-1 subtype. This may explain the increased potency in the acute treatment. If WA435 is a sigma-1 agonist, the 24 hour continuous exposure would allow continuous occupancy of the sigma-1 receptor, activation of which is known to promote cell survival (Hayashi and Su, 2007; Wu and Bowen, 2008). This could therefore compete with the cytotoxic effect of sigma-2 receptor activation. In the acute treatment, all ligand would be washed out of sigma-1 receptors and this pro-survival pathway would no longer be activated. Whether covalent attachment to the receptor via the 6-isothiocyanato moiety is key to cytotoxicity will require further study.

We have recently shown the ability of a novel SN79 derivative, CM764, to induce a non-toxic, non-zero effect through the sigma-2 receptor, comprised in part by a stimulation of MTT reduction (Nicholson, et al. 2016). In the current series, five of the thirteen SN79 derivatives shared this CM764-like phenotype with respect to stimulation of MTT reduction. While it is difficult to determine a very clear relationship between structure and ability to increase MTT reduction in SK-N-SH neuroblastoma cells, our data show some trends. High sigma-2 receptor affinity was a common factor for all ligands with this function. Neither R-group alone nor X-group alone could explain the relationship between ligand structure and the ability to induce increased MTT reduction, as compounds representing all X- substitutions and R- substitutions (with the exception of –NCS) caused MTT stimulation. Furthermore, there are also "neutral" compounds. For example, SN79 (X=O, R=$COCH_3$) itself neither kills cells nor induces significant stimulation of metabolism at 30 μM (Nicholson et al., 2016). In fact, 30 μM SN79 was able to completely attenuate the metabolic stimulation induced by 10 μM CM764. This, along with complete blockade by a low, sub-cytotoxic dose (300 nM) of the highly sigma-2 receptor-selective CM572, contributed to supporting evidence that the stimulatory effect is

mediated by sigma-2 receptors (Nicholson et al., 2016). Also, the only difference between the neutral SN79 and metabolic stimulative CM458 and CM571 is NO_2 or NH_2 in the 6-position, respectively, vs. $COCH_3$. Thus, subtle changes in structure have marked effects on compound efficacy. It seems probable that these phenotypes derive from a combinatorial effect. The X-group and R-group are conjugated in the series investigated in this study, and therefore both positions could contribute to the development of this stimulative function. In addition, with CM764, X= O, R=$COCH_3$ and there is an amine moiety in the fluorophenyl ring, indicating that modifications of the other ring system also affect the stimulatory efficacy. Further studies on the combinatorial effects and electron density changes with additional X-group and R-group substitutions may help elucidate this relationship.

The results of this study show that sigma-2 receptor ligands in the SN79-related platform can be divided into two activity classes: those that induce programmed cell death and those that stimulate MTT reduction (metabolic stimulation). This is summarized in Figure 9. A third class may be those compounds that are neutral and possibly antagonists. We have postulated that the metabolic stimulation effect (as first characterized in detail with CM764; Nicholson et al., 2016) may be related to a survival role, consistent with the upregulation of sigma-2 receptors in cancer cells, and involved in the enhancement of tumor cell adaptation in hypoxic environment. It is not yet clear how the sigma-2 receptor mediates both induction of apoptosis and this non-toxic effect on metabolism. SN79 analogs will be helpful in resolving this issue.

Authorship Contributions

Participated in research design: Nicholson, Comeau, Bowen

Conducted experiments: Nicholson, Comeau

Contributed new reagents or analytic tools: Alsharif, Mesangeau, Intagliata, Mottinelli, McCurdy

Performed data analysis: Nicholson, Comeau

Wrote or contributed to the writing of the manuscript: Nicholson, Bowen

Wrote or contributed to supplemental materials: Intagliata, Mottinelli, McCurdy

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Footnotes

Portions of this work have been previously presented in abstract form: Hilary Elaine Nicholson, Walid Alsharif, Christopher R. McCurdy, and Wayne D. Bowen. Evaluation of structural changes in SN79-derived sigma-2 receptor modulators: Effect on apoptotic efficacy in SK-N-SH neuroblastoma. [abstract]. In: Proceedings of the 106th Annual Meeting of the American Association for Cancer Research; 2015 Apr 18-22; Philadelphia, PA. Philadelphia (PA): AACR; Cancer Res 2015;75(15 Suppl): Abstract #2440. doi:10.1158/1538-7445.AM2015-2440 (http://cancerres.aacrjournals.org/content/75/15_Supplement/2440.abstract)

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Legends for Figures

Figure 1. **Effect of X-group substitutions on sigma-1 receptor binding affinity.** Affinity at sigma-1 receptors was determined by competition against [^{3}H](+)-pentazocine as described in Materials and Methods. Ki values for sigma-1 receptor binding of R=NH_2 substituted ligands were determined to be 15.5 ± 2.4 nM, 2987.7 ± 506.9 nM, and 15.6 ± 2.8 nM for CM571 (X=O, R=NH_2), WA403 (X=NMe, R=NH_2), and WA416 (X=S, R=NH_2) substitutions, respectively. This series dramatically demonstrates the general loss of sigma-1 receptor affinity for X=NMe substituted ligands compared to other X-group substitutions. Competition curves shown are an average of 3 independent experiments for CM571, 5 independent experiments for WA403, and 3 independent experiments for WA416. All experiments were performed in duplicate. Ki values reported are an average of the individual Ki value from each independent experiment, ± S.D.

Figure 2. **Effect of R-group substitutions on sigma-1 receptor binding affinity.** Affinity at sigma-1 receptors was determined by competition against [^{3}H](+)-pentazocine as described in Materials and Methods. Sigma-1 receptor binding affinity was significantly decreased when R=NCS for all X-group substitutions. This pattern is most clearly illustrated for X=O substituted ligands, with CM572 (X=O, R=NCS) showing at least a 350-fold loss in sigma-1 receptor binding affinity as compared to other R-group substitutions. Ki values for X=O substituted ligands at sigma-1 receptors were determined to be 28.03 ± 3.39* nM, >10,000 nM, 22.2 ± 5.3 nM, and 15.5 ± 2.4 nM for SN79 (X=O, R=$COCH_3$), CM572 (X=O, R=NCS), CM458 (X=O, R=NO_2), and CM571 (X=O, R=NH_2), respectively. An accurate Ki value for CM572 (X=O, R=NCS) at sigma-1 receptors could not be determined due to insolubility above 1 mM, however

~50% of [^{3}H](+)-pentazocine binding sites remained with 10,000 nM CM572 present. Competition curves shown are an average of 4 independent experiments for SN79, 2 independent experiments for CM572, 3 independent experiments for CM458, and 3 independent experiments for CM571. All experiments were performed in duplicate. Ki values reported are an average of the individual Ki value from each independent experiment, ± S.D. (*Kaushal et al., 2011)

Figure 3. **Effect of X-group substitutions on sigma-2 receptor binding affinity.** Affinity at sigma-2 receptors was determined by competition against [^{3}H]DTG as described in Materials and Methods. Generally, X-group substitutions with sulfur resulted in ligands with very high sigma-2 receptor binding affinity, ranging between 2-4 nM Ki values. This trend generally held true across R-group changes, and was most drastically demonstrated by the WA435 (X=S, R=NCS) and WA416 (X=S, R=NH_2) comparisons depicted here. For R=NCS (left panel), WA435 (X=S, R=NCS) showed at least a 7-fold higher binding affinity as compared to the other X-group substituted ligands. Similarly, for R=NH_2 (right panel), WA416 (X=S, R=NH_2) showed at least a 4.5-fold higher binding affinity compared to the other X-group substituted ligands. Competition curves shown are an average of 4 independent experiments for CM572, 6 independent experiments for WA404, 3 independent experiments for WA435, 3 independent experiments for CM571, 5 independent experiments for WA403, and 3 independent experiments for WA416. All experiments were performed in duplicate.

Figure 4. **Irreversible binding of isothiocyanate-substituted ligands at sigma-1 and sigma-2 receptors.** Rat liver membrane homogenates were treated with isothiocyanate-substituted ligands

for 60 minutes prior to washing to remove any reversibly bound ligand as described in Materials and Methods. Recovery of sigma-1 and sigma-2 binding sites was then determined using [^{3}H](+)-pentazocine for sigma-1 receptors and [^{3}H]DTG for sigma-2 receptors, as described in Materials and Methods. Data is presented as the percentage of total sigma receptors of each subtype recovered. CM572 (X=O, R=NCS) (panel A) was previously shown to irreversibly bind sigma-2 receptors but not sigma-1 receptors (Nicholson et al. 2015). WA404 (X=NMe, R=NCS) (panel B) and WA435 (X=S, R=NCS) (panel C) also showed the ability to selectively and irreversibly bind sigma-2 receptors. WA404 (X=NMe, R=NCS) demonstrated some slight ability to irreversibly bind sigma-1 receptors, but only at high concentrations. Data shown is the average of at least two independent experiments for each condition, with each experiment performed in duplicate.

Figure 5. **Effect of isothiocyanate derivatives on cell viability of SK-N-SH neuroblastoma.** SK-N-SH neuroblastoma cells were plated at 15,000 cells/well and allowed to attach overnight prior to dosing with indicated concentration of each ligand. Cells were incubated with the indicated compounds for 24 hours, prior to determination of viable cells remaining using MTT assay, as described in Materials and Methods. Data are presented as % cytotoxicity. All three isothiocyanate derivatives induced dose-dependent cell death. EC_{50} values were 7.6 ± 1.7 μM*, 32.76 ± 1.05 μM, and 30.35 ± 1.13 μM for CM572 (X=O, R=NCS), WA435 (X=S, R=NCS), and WA404 (X=NMe, R=NCS), respectively. Dose-response curves shown are an average of 3 independent experiments each ligand, with each experiment performed using 5 replicates per condition. EC_{50} values were determined from average cytotoxicity. (*Nicholson et al., 2015)

Figure 6. **Comparison of acute and continuous exposure to isothiocyanate substituted ligands on cell viability of SK-N-SH neuroblastoma.** Cells were plated at 15,000 cells/well and allowed to attach overnight prior to dosing with indicated concentration of each ligand. Cells were incubated with indicated ligand continuously for 24 hours (black bars) or cells were treated with ligand for 60 minutes followed by extensive washing with fresh media and 24 hour incubation in ligand-free media (white bars). At the end of the 24 hour period, viable cells remaining were determined by MTT assay. Treatment with all three isothiocyanate derivatives resulted in either comparable or greater levels of cell death in the acute exposure experiment as in the 24 hour continuous exposure ($p=0.13$, 0.056, and 0.003 for CM572, WA404, and WA435 comparisons of the two exposure conditions, Student's t-test). These data indicate that the isothiocyanate substituted ligands continue to induce cell death as a result of their capacity to irreversibly bind sigma-2 receptors even after free ligand is removed. Results are presented as the percentage of cells killed in response to each treatment compared to untreated control cells. Data is presented as an average of 3 independent experiments for each condition for each ligand, ± S.D. All experiments were performed with 5 replicates per condition.

Figure 7. **Binding of phenyl isothiocyanate at sigma receptors and effect on viability of SK-N-SH neuroblastoma.** A) Sigma-1 and sigma-2 receptor binding was determined by competition of phenyl isothiocyanate with [^{3}H](+)-pentazocine or [^{3}H]DTG in the presence of unlabeled (+)-pentazocine, respectively. No significant binding at sigma-1 or sigma-2 receptors was observed upon competition with phenyl isothiocyanate. Experiments were performed in

duplicate. B) Cells were plated at 15,000 cells/well and allowed to attach overnight prior to treatment with the indicated concentrations of phenyl isothiocyanate for 24 h, followed by MTT assay. No significant cytotoxicity was observed in 2% serum conditions, and only very high concentrations of phenyl isothiocyanate reached significance in 10% serum conditions as compared to untreated cells (two-way ANOVA Dunnett's multiple comparisons post-hoc test *$p<0.05$ compared to untreated cells)

Figure 8. **Ability of SN79 derivatives to increase reduction of MTT in SK-N-SH neuroblastoma.** Cells were plated at 15,000 cells/well and allowed to attach overnight prior to treatment with indicated ligand and concentration for 24 h, followed by MTT assay. Five out of twelve SN79 derivatives in this series demonstrated the ability to induce a significant increase in the amount of MTT reduced as compared to untreated cells (two-way ANOVA overall $p<0.0001$. Dunnett's multiple comparisons post-hoc test *$p<0.05$, ***$p<0.001$, ****$p<0.0001$ compared to untreated cells). All R-group substitutions were represented except R=NCS, which is consistent with the ability of R=NCS ligands to induce cell death. All X-group substitutions were also represented in this subset, and all ligands demonstrated high sigma-2 receptor binding affinity. Results are presented as the percent change in MTT reduction as compared to an untreated control, which was normalized to zero. Data are presented as an average ± S.D. of at least three independent experiments for each condition, with each experiment performed using five replicates per condition.

Figure 9. **Divergent effects of SN79 analogs.** Diagram shows structural relationship of compounds examined in this study and the effects that are elicited. SN79 is shown as the parent compound. The heterocyclic X-atom was varied as O (benzoxazolone), N-methyl (N-methylbenzimidazolone), and S (benzothiazolone). The R-group in the 6-position was substituted with isothiocyanate, nitro, amine, methylketone, or fluorine. Only compounds with R=NCS, regardless of heterocycle, were able to potently induce programmed cell death. Several compounds with the other 6-position substituents were metabolic stimulators, regardless of heterocycle. Other non-isothiocyanates, including SN79 itself, were neutral, producing neither programmed cell death nor metabolic stimulation at concentrations up to 30 μM. The dotted line linking the groups indicates that both effects appear to be mediated by sigma-2R/TMEM97.

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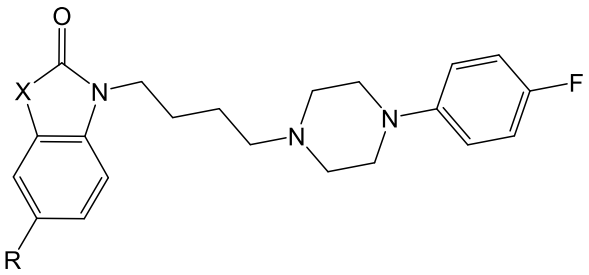
Tables

Table 1:

Binding affinities of SN79 derivatives at sigma-1 and sigma-2 receptors.



			Sigma binding affinities (Ki ± SD) (nM)		
			[^{3}H](+)-pentazocine	[^{3}H]DTG	
Ligand	**X**	**R**	**sigma-1**	**sigma-2**	**s1/s2**
SN79	O	$COCH_3$	28.0 ± 3.39*	6.89 ± 0.09*	4.06
CM572	O	NCS	≥10,000†	14.6 ± 7.0†	>685
CM458	O	NO_2	22.2 ± 5.3	0.56 ± 0.38	39.6
CM571	O	NH_2	15.5 ± 2.4	21.7 ± 5.3	0.7
NF7	NMe	$COCH_3$	183 ± 13.9	20.6 ± 2.4	8.9
WA404	NMe	NCS	449 ± 44.4	36.3 ± 7.8	12.4
WA402	NMe	NO_2	20.3 ± 6.5	7.4 ± 3.0	2.7
WA403	NMe	NH_2	2,988 ± 507	17.9 ± 8.0	167
WA504	S	$COCH_3$	8.1 ± 2.3	2.5 ± 2.7	3.2
WA435	S	NCS	56.9 ± 10.2	2.0 ± 1.5	28.5
WA413	S	NO_2	6.1 ± 1.0	3.2 ± 0.3	1.9
WA416	S	NH_2	15.6 ± 2.8	3.9 ± 0.5	4.0
WA379	NMe	F	101 ± 10.3	6.1 ± 1.7	16.5

Radioligand competition binding assays were performed using [^{3}H](+)-pentazocine to measure sigma-1 receptor and [^{3}H]DTG in the presence of unlabeled (+)-pentazocine to measure sigma-2

receptors as described in Experimental Section. Single-position changes to the core structure of SN79 resulted in drastic changes in affinity and selectivity at sigma-1 and sigma-2 receptors. Generally, changes in the X-position determined selectivity, with –S- substitutions resulting in reduced selectivity and –O- substitutions resulting in increased selectivity for the sigma-2 receptor. R-group substitutions did not demonstrate a discernably consistent pattern across the ligand series. An accurate Ki value could not be determined for CM572 (X=O, R=NCS) at sigma-1 receptors due to insolubility above 1 mM, however ~50% of total sigma-1 receptors were bound by 10,000 nM CM572 at compared to total available sigma-1 receptor binding capacity as measured by [^{3}H](+)-pentazocine alone. Results are presented as an average Ki values from at least three independent experiments for each ligand (except for CM572 at sigma-1 receptors), ± S.D. All experiments were performed in duplicate. *(Kaushal et al., 2011) †(Nicholson et al., 2015)

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Figures

Figure 1

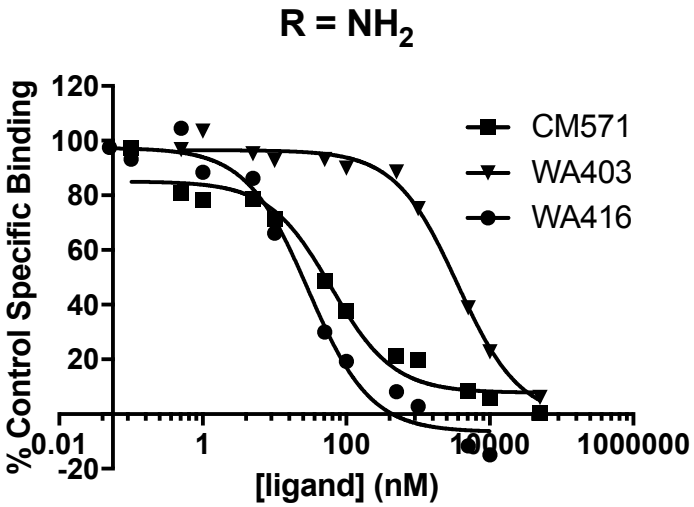

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Figure 2

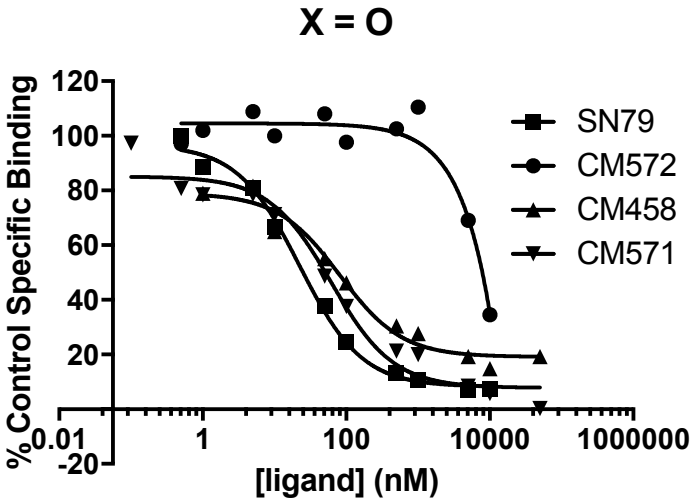

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Figure 3

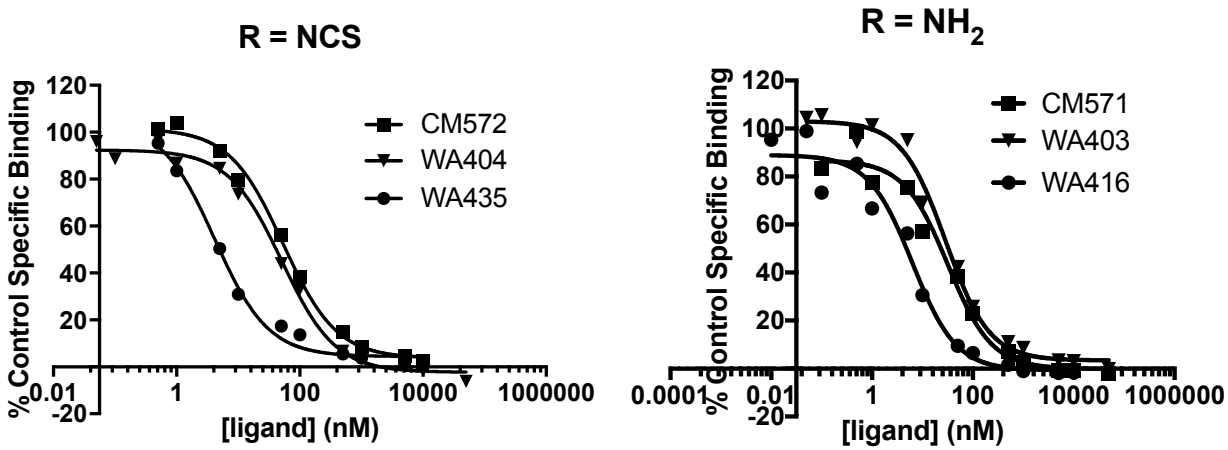

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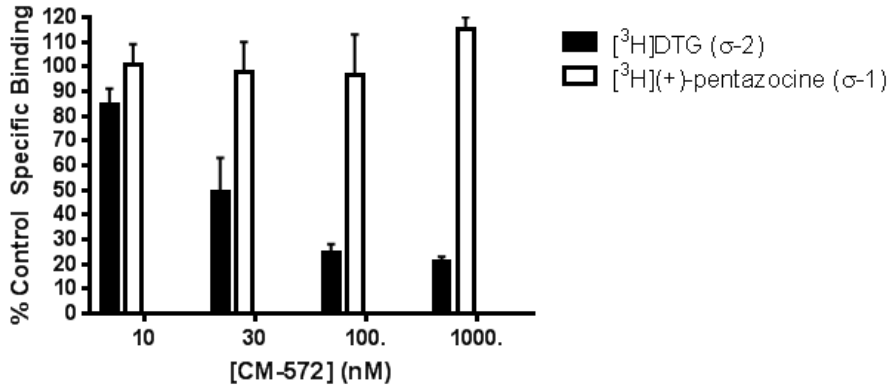
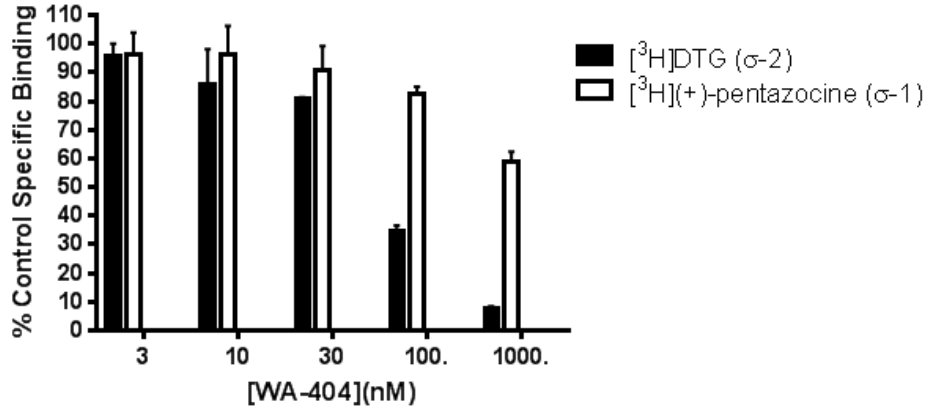
Figure 4

A.



B.



C.

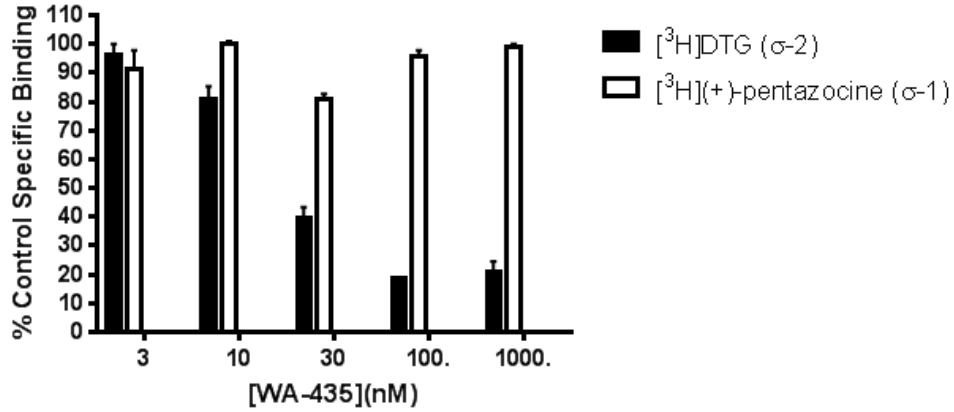


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Figure 5

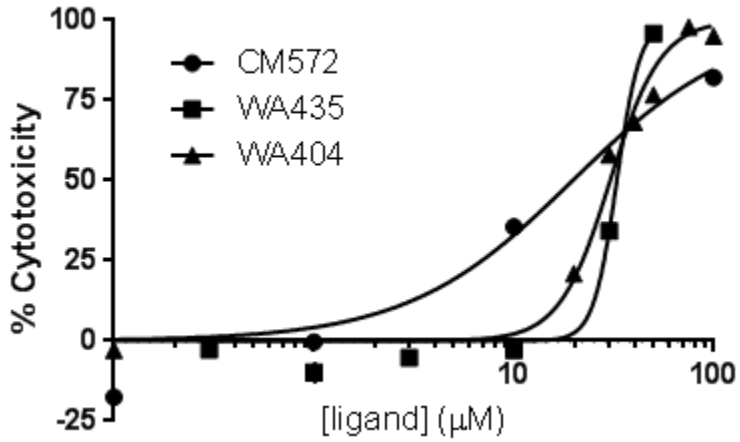

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Figure 6

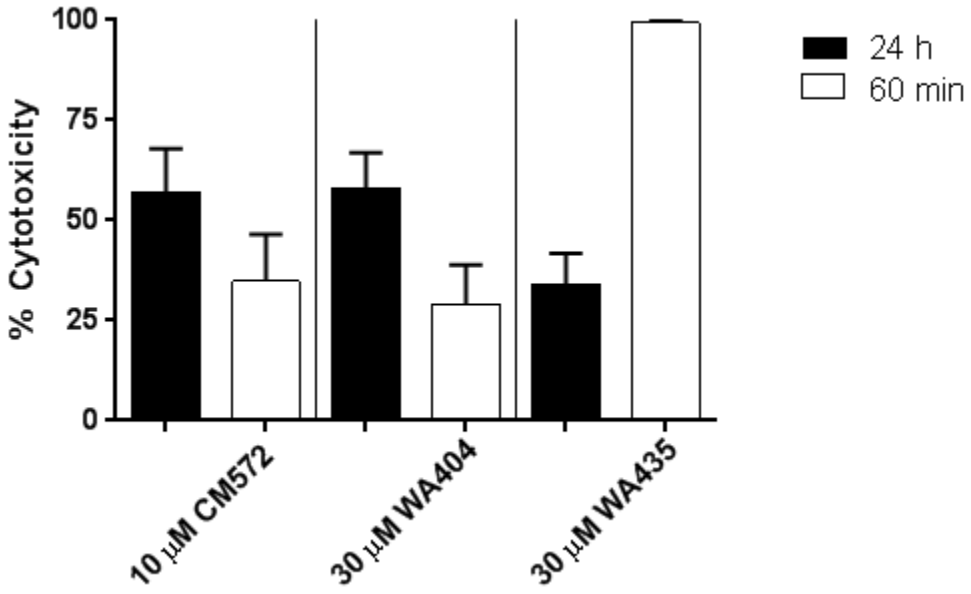

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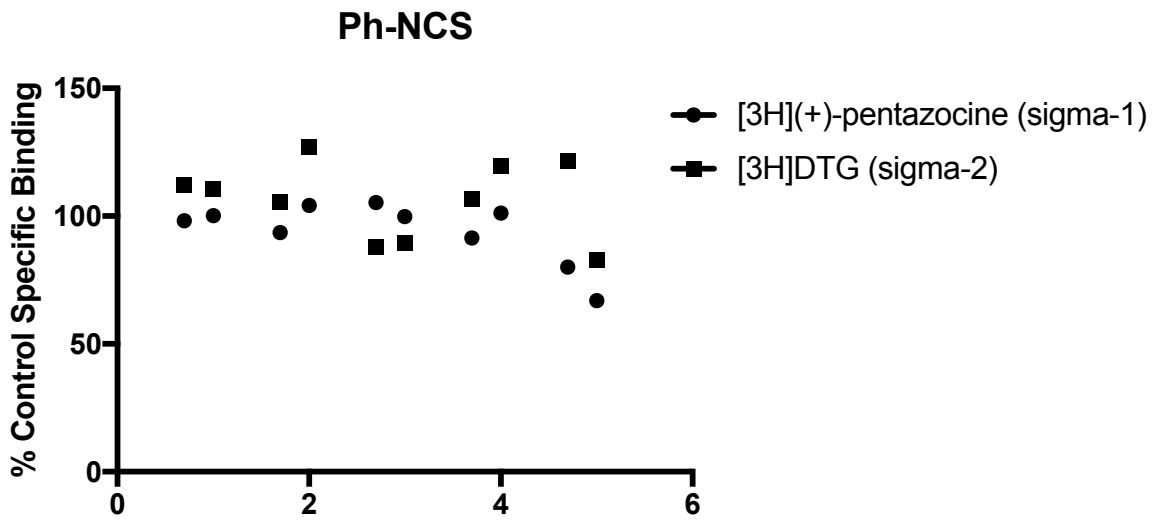
Figure 7

A



B

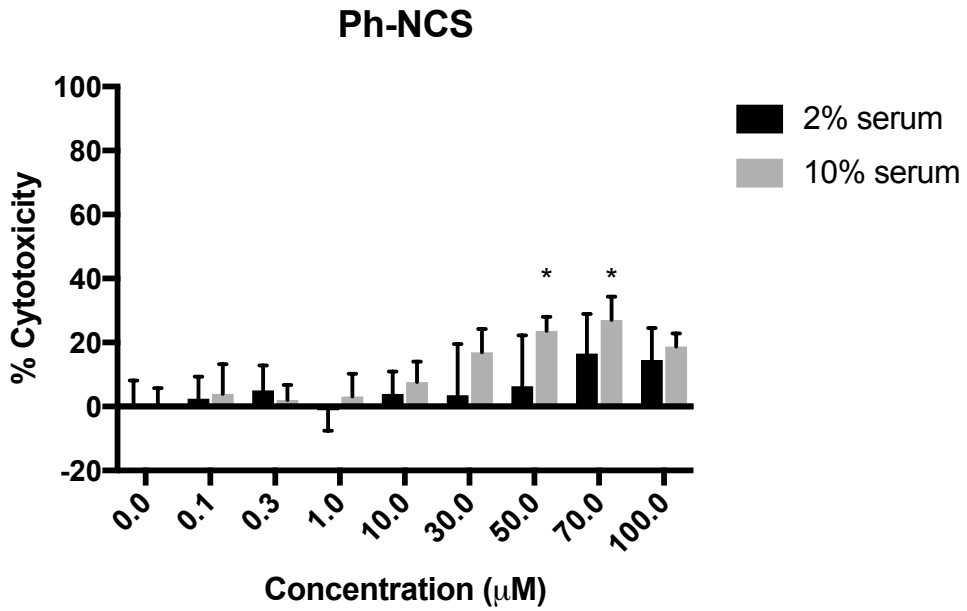


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Figure 8

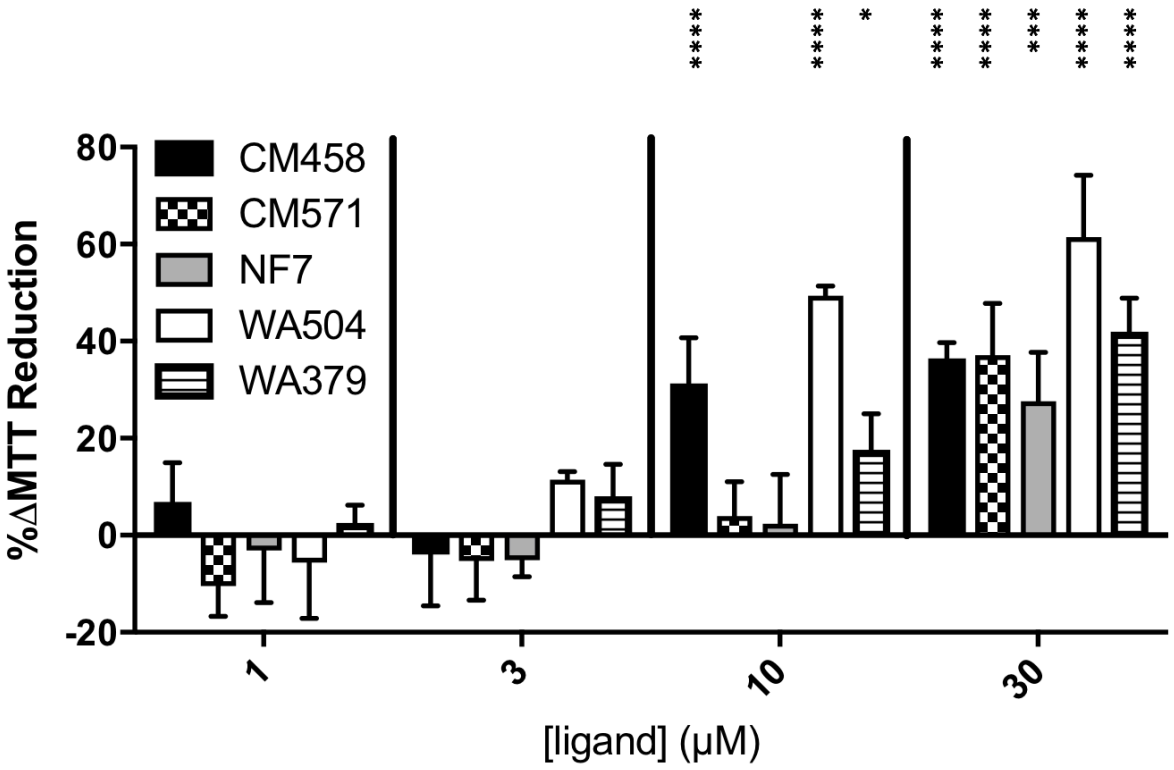

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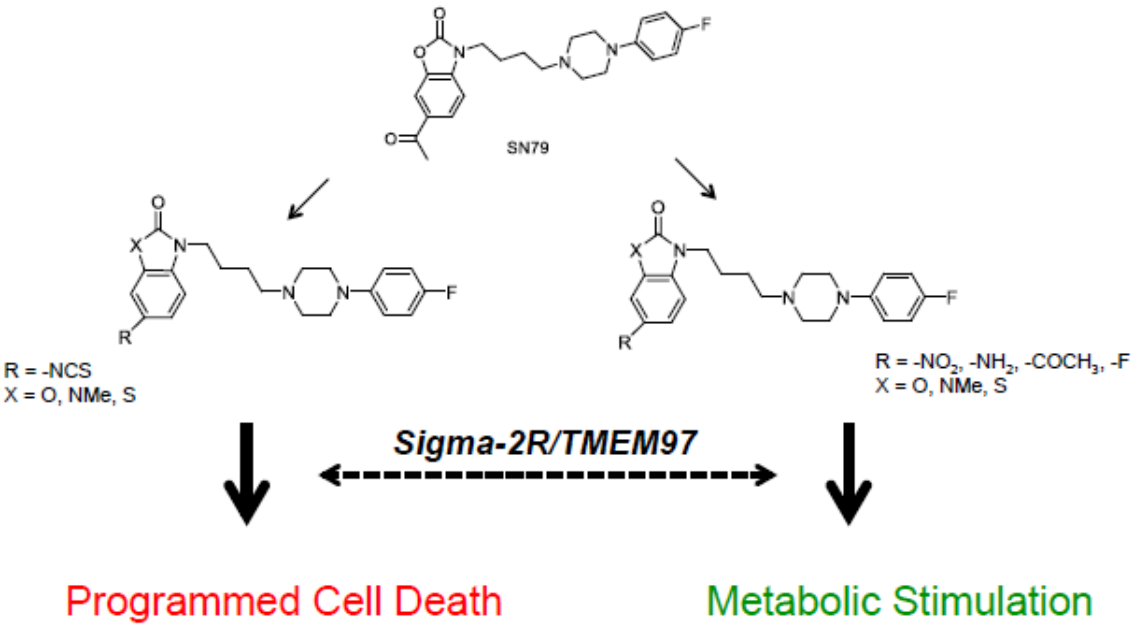
Figure 9

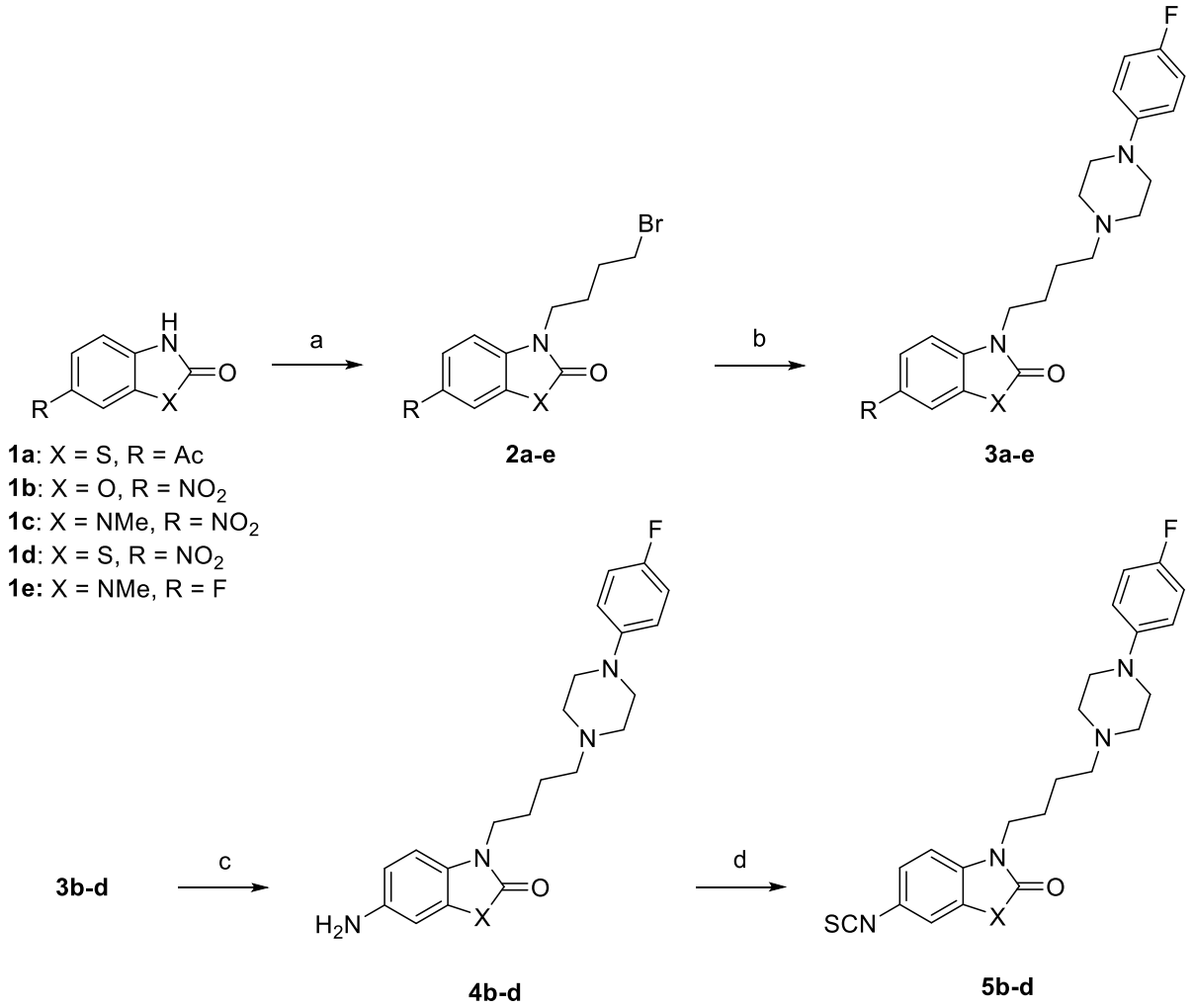


The Journal of Pharmacology and Experimental Therapeutics (JPET #253484)

Title: Divergent Cytotoxic and Metabolically Stimulative Functions of Sigma-2 Receptors: Structure-activity Relationships of 6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[d]oxazol-2(3H)-one (SN79) Derivatives

Authors: Hilary E. Nicholson, Walid F. Alsharif, Anthony B. Comeau, Christophe Mesangeau, Sebastiano Intagliata, Marco Mottinelli, Christopher R. McCurdy, and Wayne D. Bowen*

Chemistry



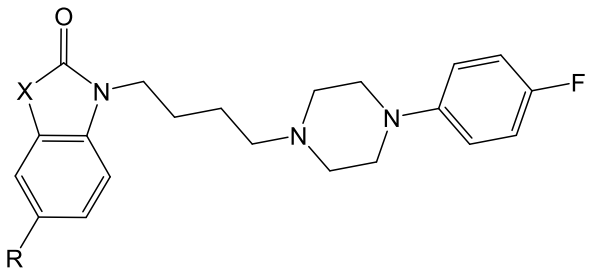
Scheme 1. Synthesis of final compounds **3a-e**, **4b-d** and **5b-d**. Reagents and conditions: (a) 1,4-dibromobutane, K_2CO_3, DMF, 60 °C, 2-3 hr, 66-83%; (b) 1-(4-fluorophenyl)piperazine, K_2CO_3, DMF, 60 °C, 2-4 hr, 63-88%; (c) H_2 (20-50 psi), 10% Pd/C, MeOH or THF/MeOH (1:1), 1-2 hr, 75-87%; (d) thiophosgene, TEA, DCM, 0 °C, 2h, 53% or 1,1′-thiocarbonyldiimidazole, DMF, 5 min at 50 °C then 30 min at room temperature, 54-60%.

Synthesis of **SN79** and **NF7** have been carried out as already described in refences number 1 and 2, respectively. Compounds **3a-g**, **4b-d** and **5b-d** were synthesized according to scheme 1.

Commercially available compounds **1a-e** were treated with 1,4-dibromobutane under base catalyzed conditions to give the bromoalkyl intermediates **2a-e**, which were subsequently coupled with 1-(4-fluorophenyl)piperazine in the presence of potassium carbonate to give the final compounds **3a-e**. Nitro derivatives **3b-d** were further reduced to the corresponding amines **4b-d**, which were then converted into the final isothiocyanates **5b-d** (**Scheme 1**).

Table 1:

Binding affinities of SN79 derivatives at sigma-1 and sigma-2 receptors.



				Sigma binding affinities (Ki ± SD) (nM)		
				[^{3}H](+)-pentazocine	[^{3}H]DTG	
Compound	**Name**	**X**	**R**	**sigma-1**	**sigma-2**	**s1/s2**
	SN79	O	$COCH_3$	28.0 ± 3.39*	6.89 ± 0.09*	4.06
	NF7	NMe	$COCH_3$	183 ± 13.9	20.6 ± 2.4	8.9
3a†	WA504	S	$COCH_3$	8.1 ± 2.3	2.5 ± 2.7	3.2
3b†	CM458	O	NO_2	22.2 ± 5.3	0.56 ± 0.38	39.6
3c	WA402	NMe	NO_2	20.3 ± 6.5	7.4 ± 3.0	2.7
3d	WA413	S	NO_2	6.1 ± 1.0	3.2 ± 0.3	1.9
3e	WA379	NMe	F	101 ± 10.3	6.1 ± 1.7	16.5
4b†	CM571	O	NH_2	15.5 ± 2.4	21.7 ± 5.3	0.7
4c	WA403	NMe	NH_2	2988 ± 507	17.9 ± 8.0	167
4d	WA416	S	NH_2	15.6 ± 2.8	3.9 ± 0.5	4.0
5b‡	CM572	O	NCS	≥10,000§	14.6 ± 7.0§	>685
5c	WA404	NMe	NCS	449 ± 44.4	36.3 ± 7.8	12.4
5d	WA435	S	NCS	56.9 ± 10.2	2.0 ± 1.5	28.5

†Compound was tested as hydrochloride salt; ‡Compound was tested as dioxalate salt; *(Kaushal et al. 2011) §(Nicholson et al. 2015)

Experimental

Chemistry

Reagents and starting materials were obtained from commercial suppliers and were used without purification. Precoated silica gel 60 F254 aluminum backed plates from EMD or GF Uniplates from Analtech were used for thin-layer chromatography (TLC). Column chromatography was performed 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 or a Bruker DRX500 at 500 and 125 MHz, respectively. The mass spectra (MS) were recorded on a Waters Aquity Ultra Performance LC with ZQ detector in ESI mode. Analytical HPLC was performed on an automated Waters Alliance system equipped with a XBridge® C18 2.5mm (4.6 x 75 mm i.d., 2.5 μm) or XBridge® C18 5mm (4.6 x 75 mm i.d., 5μm) column, with a flow rate of 1 ml/min.; λ_{max} = 254 nm; mobile phase A: CH_3CN; mobile phase B: H_2O (0.2% triethylamine) linear gradient in 12 min. High Resolution Mass Spectra (HRMS) were recorded on a Waters Micromass Q-Tof Micro mass spectrometer with a lock spray source. 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 10.0, 13.0 or 14.0). The overall yields, ^{1}H and ^{13}C NMR data for final compounds are reported in its free base form unless otherwise specified.

6-acetyl-3-(4-bromobutyl)benzo[*d*]thiazol-2(3*H*)-one (2a, WA503)

To a solution of **1a** (0.69 g, 3.55 mmol) in 5 mL of DMF were added potassium carbonate (1.50 g, 10.90 mmol) and 1,4-dibromobutane (3.00 g, 13.90 mmol). The reaction mixture was heated at 60 °C for 2 h, then the mixture was poured into water and extracted with EtOAc. The organic phase was dried over sodium sulfate, filtered and evaporated under vacuum. The crude compound was purified by column chromatography (EtOAc/hexane 2:8) to afford **2a** as a pale yellow oil (0.85 g, 73%). ^{1}H NMR (400 MHz, MeOD) δ 8.14 (d, J = 1.8 Hz, 1H), 7.99 (dd, J = 8.5, 1.8 Hz, 1H), 7.33 (d, J = 8.5 Hz, 1H), 4.03 (dd, J = 7.8, 5.2 Hz, 2H), 3.52 – 3.44 (m, 2H), 2.59 (s, 3H), 1.93 – 1.84 (m, 4H). ^{13}C NMR (101 MHz, MeOD) δ 197.1, 170.4, 140.7, 132.3, 127.3, 123.1, 122.6, 110.5, 41.6, 32.2, 29.5, 25.9, 25.2. MS (ESI) *m/z* 328 $[M+H]^+$ (^{79}Br) and 330 $[M+H]^+$ (^{81}Br).

3-(4-bromobutyl)-6-nitrobenzo[*d*]oxazol-2(3*H*)-one (2b, CM187)

To a solution of **1b** (3.00 g, 22.20 mmol) in 30 mL DMF were added potassium carbonate (9.20 g, 66.60 mmol) and 1,4-dibromobutane (21.00 g, 177.60 mmol). The reaction mixture was heated at 65 °C for 3 hr, then the mixture was poured into water and extracted with EtOAc. The organic phase was dried over sodium sulfate, filtered and evaporated under vacuum. The crude compound was

purified by column chromatography (EtOAc/petroleum ether 2:8) to afford **2b** as yellow solid (4.36 g, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.22 (dd, J = 8.4, 2 Hz, 1H), 8.10 (d, J = 2 Hz, 1H), 7.12 (d, J = 8.8 Hz, 1H), 3.95 (t, J = 6.4 Hz, 2H), 3.47 (t, J = 6.0 Hz, 2H), 1.95-2.09 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 153.9, 143.3, 141.9, 136.4, 120.9, 107.4, 106.3, 41.9, 32.3, 29.2, 26.2. MS (ESI^+) m/z 315 $[M+1]^+$ (^{79}Br), 317 $[M+1]^+$ (^{81}Br).

1-(4-bromobutyl)-3-methyl-5-nitro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one (2c, WA401)

To a solution of **1c** (0.6 g, 3.11 mmol) in 10 mL DMF were added potassium carbonate (1.28 g, 9.27 mmol) and 1,4-dibromobutane (2.68 g, 12.40 mmol). The reaction mixture was heated at 65 °C for 2 hr, then the mixture was poured into water and extracted with EtOAc. The organic phase was dried over sodium sulfate, filtered and evaporated under vacuum. The crude compound was purified by column chromatography (EtOAc/Hexane 1:1) to afford **2c** as yellow oil (0.78 g, 76%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.12 (q, J = 2.7 Hz, 1H), 8.04 – 8.00 (m, 1H), 7.33 (dd, J = 8.7, 2.5 Hz, 1H), 3.95 (ddt, J = 7.2, 4.8, 2.3 Hz, 2H), 3.56 (td, J = 6.2, 2.3 Hz, 2H), 3.41 – 3.37 (m, 3H), 1.86 – 1.74 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.5, 142.1, 135.6, 129.3, 118.3, 107.9, 103.8, 40.3, 35.0, 29.8, 27.9, 27.0. MS (ESI^+) m/z 351 $[M+23]^+$.

3-(4-bromobutyl)-6-nitrobenzo[*d*]thiazol-2(3*H*)-one (2d, WA408)

To a solution of **1d** (3.0 g, 15.3 mmol) in 35 mL DMF were added potassium carbonate (6.30 g, 45.65 mmol) and 1,4-dibromobutane (13.20 g, 61.1 mmol). The reaction mixture was heated at 65 °C for 2 hr, then the mixture was poured into water and extracted with EtOAc. The organic phase was dried over sodium sulfate, filtered and evaporated under vacuum. The crude compound was purified by column chromatography (EtOAc/Hexane 1:1) to afford **2d** as yellow oil (3.30 g, 66%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.68 (d, J = 2.4 Hz, 1H), 8.22 (dd, J = 9.0, 2.5 Hz, 1H), 7.59 (d, J = 9.0 Hz, 1H), 4.03 (t, J = 6.9 Hz, 2H), 3.54 (t, J = 6.4 Hz, 2H), 1.91 – 1.70 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.9, 143.2, 142.5, 123.2, 123.1, 119.7, 111.8, 42.4, 34.8, 29.8, 26.3. MS (ESI^+) m/z 353 $[M+23]^+$.

1-(4-bromobutyl)-5-fluoro-3-methyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one (2e, WA377)

To a solution of **1e** (0.93 g, 5.60 mmol) in 15 mL DMF were added potassium carbonate (2.32 g, 16.81 mmol) and 1,4-dibromobutane (4.84 g, 22.40 mmol). The reaction mixture was heated at 65 °C for 2 hr, then the mixture was poured into water and extracted with EtOAc. The organic phase was dried over sodium sulfate, filtered and evaporated under vacuum. The crude compound was purified by column chromatography (EtOAc/Hexane 1:1) to afford **2e** as white solid (1.40 g, 83%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.16 (dd, J = 8.6, 4.5 Hz, 1H), 7.10 (dd, J = 9.1, 2.5 Hz, 1H), 6.85 (ddd, J =

10.2, 8.6, 2.5 Hz, 1H), 3.83 (t, *J* = 6.4 Hz, 2H), 3.54 (t, *J* = 6.2 Hz, 2H), 3.30 (s, 3H), 1.76 (dddd, *J* = 15.4, 10.7, 8.0, 4.6 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 158.4 (d, *J* = 232.3 Hz), 154.4, 131.0 (d, *J* = 10.1 Hz), 125.6, 108.6 (d, *J* = 10.1 Hz), 107.3 (d, *J* = 30.3 Hz), 96.7 (d, *J* = 30.3 Hz), 40.0, 34.9, 29.8, 27.6, 27.0. MS (ESI$^+$) *m/z* 324 $[M+23]^+$.

6-acetyl-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[*d*]thiazol-2(3*H*)-one dihydrochloride (3a, WA504)

K_2CO_3 (0.86 g, 6.23 mmol) and 1-(4-fluorophenyl)piperazine (0.33 g, 1.83 mmol) were added, under mechanical stirring, to a solution of **2a** (0.50 g, 1.52 mmol) in 5 mL of anhydrous DMF. The reaction mixture was heated at 60 °C for 3 h. After cooling, the mixture was poured into water and extracted with EtOAc. The organic phase was dried over sodium sulfate, filtered and evaporated under vacuum. The crude compound was purified by column chromatography (EtOAc/hexane 1:1) to afford **3a**, which was readily converted into the hydrochloride salt by addition of HCl/dioxane and isolated as a white solid (0.45 g, 59%). ^{1}H NMR (400 MHz, DMSO) δ 12.48 (s, 1H), 11.53 (s, 1H), 8.31 (s, 1H), 7.95 (d, *J* = 8.5 Hz, 1H), 7.54 (d, *J* = 8.5 Hz, 1H), 7.07 (dt, *J* = 12.0, 8.3 Hz, 4H), 4.02 (t, *J* = 6.5 Hz, 2H), 3.70 (d, *J* = 12.1 Hz, 2H), 3.50 (d, *J* = 11.6 Hz, 2H), 3.23 (t, *J* = 12.0 Hz, 2H), 3.13 (dd, *J* = 10.5, 6.3 Hz, 4H), 2.54 (t, *J* = 13.6 Hz, 3H), 1.89 – 1.77 (m, 2H), 1.76 – 1.60 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 196.7, 169.9, 157.3 (d, *J* = 238.4 Hz), 146.5, 140.9, 132.5, 127.7, 124.1, 122.4, 118.6 (d, *J* = 10.1 Hz), 116.0 (d, *J* = 22.2 Hz), 111.7, 55.2, 50.8, 46.6, 42.4, 27.1, 25.1, 20.7. MS (ESI$^+$) *m/z* 429 $[M+H]^+$.

3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-6-nitrobenzo[*d*]oxazol-2(3*H*)-one dihydrochloride (3b, CM458)

K_2CO_3 (0.11 g, 0.77 mmol) and 1-(4-fluorophenyl)piperazine (0.04 g, 0.19 mmol) were added, under mechanical stirring, to a solution of **2b** (0.06 g, 0.19 mmol) in 4 mL of anhydrous DMF. The reaction mixture was heated at 60 °C for 3 h. After cooling, the mixture was poured into water and extracted with EtOAc. The organic phase was dried over sodium sulfate, filtered and evaporated under vacuum. The crude compound was purified by column chromatography (DCM/MeOH 95:5) to afford **3b**, which was readily converted into the hydrochloride salt by addition of HCl/dioxane and isolated as a yellow solid (0.05 g, 63%). ^{1}H NMR (DMSO-d_6): δ 11.52 (br s, 1H), 8.57 (br s, 1H), 8.24 (d, *J*= 2.0 Hz, 1H), 8.20 (dd, J= 8.6, 2.0 Hz, 1H), 7.63 (d, *J*= 8.6 Hz, 1H), 7.11-7.01 (m, 4H), 3.93 (d, *J* = 6.7 Hz, 2H), 3.69 (d, *J* = 12.5 Hz, 2H), 3.51 (d, *J* = 11.5 Hz, 2H), 3.25-3.10 (m, 6H), 1.84-1.78 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 156.7 (d, *J* = 235.8 Hz), 153.8, 146.1 (d, *J* = 2.0 Hz), 142.3, 141.5, 137.2, 120.8, 118.0 (d, *J* = 7.7 Hz), 115.5 (d, *J* = 21.9 Hz), 109.0, 105.6, 54.7, 50.4, 46.1, 41.8, 24.5, 20.1.

HRMS Calcd for $C_{21}H_{24}FN_4O_4$ $[M+H]^+$ 415.1782, found 415.1787. Anal. calcd for $C_{21}H_{25}Cl_2FN_4O_4$: C, 51.75; H, 5.17; N, 11.50. Found: C, 51.60; H, 4.91; N, 11.37.

1-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-3-methyl-5-nitro-1,3-dihydro-2*H*benzo[*d*]imidazol-2-one (3c, WA402)

K_2CO_3 (2.32 g, 16.81 mmol) and 1-(4-fluorophenyl)piperazine (2.32 g, 16.81 mmol) were added, under mechanical stirring, to a solution of **2c** (0.94 g, 5.60 mmol) in 15 mL of anhydrous DMF. The reaction mixture was heated at 65 °C for 2 h. After cooling, the mixture was poured into water and extracted with EtOAc. The organic phase was dried over sodium sulfate, filtered and evaporated under vacuum. The crude compound was purified by column chromatography (EtOAc/Hexane 1:1) to afford **3c** as yellow oil (0.91 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (ddd, *J* = 8.6, 5.0, 2.2 Hz, 1H), 7.90 (dd, *J* = 7.0, 2.2 Hz, 1H), 7.04 (dd, *J* = 12.7, 8.6 Hz, 1H), 6.98 – 6.91 (m, 2H), 6.90 – 6.83 (m, 2H), 3.98 (td, *J* = 7.3, 4.2 Hz, 2H), 3.49 (d, *J* = 3.6 Hz, 3H), 3.11 (dd, *J* = 6.3, 3.7 Hz, 4H), 2.59 (q, *J* = 5.4 Hz, 4H), 2.44 (q, *J* = 6.6, 6.0 Hz, 2H), 1.84 (h, *J* = 7.3 Hz, 2H), 1.61 (p, *J* = 7.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.1 (d, *J* = 242.4 Hz), 154.6, 147.9, 142.6, 135.1, 130.0, 129.2, 118.3, 117.8 (d, *J* = 7.7 Hz), 115.3 (d, *J* = 30.3 Hz), 106.5, 103.3, 57.7, 53.2, 50.1, 41.4, 27.6, 26.2, 23.9. MS (ESI^+) *m/z* 429 $[M+1]^+$.

3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-6-nitrobenzo[*d*]thiazol-2(3*H*)-one (3d, WA413)

K_2CO_3 (1.25 g, 9.05 mmol) and 1-(4-fluorophenyl)piperazine (0.65 g, 3.62 mmol) were added, under mechanical stirring, to a solution of **2d** (1.00 g, 3.02 mmol) in 15 mL of anhydrous DMF. The reaction mixture was heated at 65 °C for 4 h. After cooling, the mixture was poured into water and extracted with EtOAc. The organic phase was dried over sodium sulfate, filtered and evaporated under vacuum. The crude compound was purified by column chromatography (EtOAc/Hexane 1:1) to afford **3d** as yellow solid (1.00 g, 76%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (d, *J* = 2.3 Hz, 1H), 8.23 (dd, *J* = 8.9, 2.3 Hz, 1H), 7.21 (d, *J* = 8.9 Hz, 1H), 6.94 (t, *J* = 8.7 Hz, 2H), 6.86 (dd, *J* = 9.2, 4.4 Hz, 2H), 4.05 (t, *J* = 7.4 Hz, 2H), 3.11 (t, *J* = 4.9 Hz, 4H), 2.60 (t, *J* = 4.9 Hz, 4H), 2.46 (t, *J* = 7.2 Hz, 2H), 1.82 (q, *J* = 7.6 Hz, 2H), 1.63 (q, *J* = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.5, 157.2 (d, *J* = 252.5 Hz), 147.9 (d, *J* = 2.0 Hz), 143.3, 141.9, 123.6, 122.6, 118.7, 117.8 (d, *J* = 10.1 Hz), 115.5 (d, *J* = 20.2 Hz), 110.2, 57.5, 53.2, 50.1, 43.2, 25.4, 23.7. MS (ESI^+) *m/z* 432 $[M+H]^+$.

5-fluoro-1-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-3-methyl-1,3-dihydro-2*H*benzo[*d*]imidazol-2-one (3e, WA379)

K_2CO_3 (0.41 g, 2.98 mmol) and 1-(4-fluorophenyl)piperazine (0.23 g, 1.29 mmol) were added, under mechanical stirring, to a solution of **2e** (0.30 g, 0.99 mmol) in 10 mL of anhydrous DMF. The reaction mixture was heated at 65 °C for 3 h. After cooling, the mixture was poured into water and extracted with DCM. The organic phase was dried over magnesium sulfate, filtered and evaporated under vacuum. The crude compound was purified by column chromatography (EtOAc/Hexane 1:1) to afford **3e** as yellow oil (0.35 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.99 – 6.92 (m, 2H), 6.92 – 6.84 (m, 3H), 6.82 – 6.71 (m, 2H), 3.91 (t, *J* = 7.1 Hz, 2H), 3.39 (s, 3H), 3.15 – 3.06 (m, 4H), 2.63 – 2.54 (m, 4H), 2.48 – 2.40 (m, 2H), 1.86 – 1.74 (m, 2H), 1.67 – 1.54 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.7 (d, *J* = 232.3 Hz), 157,2 (d, *J* = 232.3 Hz), 154.7, 147.9, 130.8, 125.4, 117.8 (d, *J* = 10.1 Hz), 115.5 (d, *J* = 10.1 Hz), 107.7 (d, *J* = 10.1 Hz), 107.3 (d, *J* = 20.2 Hz), 95.9 (d, *J* = 20.2 Hz), 57.8, 53.2, 50.1, 41.0, 27.3, 26.2, 23.9. MS (ESI^+) *m/z* 402 $[M+H]^+$.

6-Amino-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[*d*]oxazol-2(3*H*)-one dihydrochloride (4b, CM571)

A solution of **3b** (0.58 g, 1.34 mmol) in 100 mL of a mixture of methanol and THF (1:1) was hydrogenated over 10% Pd/C (0.15 g) under pressure (20 psi) for 1 h. The catalyst was removed by filtration and the solvent was evaporated under vacuum. The residue was purified by column chromatography (EtOAc/MeOH 95:5) to afford **4b**, which was readily converted into the hydrochloride salt by addition of HCl/dioxane and isolated as a white solid (0.57 g, 87%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.11-10.36 (br m, 3H), 7.44 (d, *J* = 8.3 Hz, 1H), 7.37 (s, 1H), 7.23 (d, *J* = 8.3 Hz, 1H), 7.08 (t, *J* = 8.8 Hz, 2H), 7.01-6.98 (m, 2H), 3.86 (t, *J* = 6.6 Hz, 2H), 3.67 (s, 2H), 3.48 (s, 2H), 3.14-3.13 (m, 6H), 1.83-1.76 (m, 4H). ^{13}C NMR (500 MHz, DMSO-d_6): δ 156.5 (d, *J* = 235.3 Hz), 153.7, 146.4, 142.0, 129.7, 128.0, 118.1, 117.8 (d, *J* = 7.6 Hz), 115.5 (d, *J* = 21.9 Hz), 109.7, 104.7, 54.8, 50.6, 46.0, 41.3, 24.5, 20.2. HRMS calcd for $C_{21}H_{26}FN_4O_2$ $[M+H]^+$ 385.2040, found 385.2041.

5-amino-1-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-3-methyl-1,3-dihydro-2*H*benzo[*d*]imidazol-2-one (4c, WA403)

A solution of **3c** (0.60 g, 1.40 mmol) in 200 mL of methanol was hydrogenated over 10% Pd/C (0.10 g) under pressure (40 psi) for 2 h. The catalyst was removed by filtration and the solvent was evaporated under vacuum. The residue was purified by column chromatography (DCM/MeOH 95:5) to afford **4c** as a brown solid (0.45 g, 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.01 (td, *J* = 8.8, 1.9 Hz, 2H), 6.89 (ddt, *J* = 6.7, 4.6, 1.9 Hz, 2H), 6.78 (dd, *J* = 8.2, 1.7 Hz, 1H), 6.41 (d, *J* = 2.1 Hz, 1H), 6.31 (dt, *J* = 8.2, 1.9 Hz, 1H), 4.76 (s, 2H), 3.76 – 3.67 (m, 2H), 3.21 (d, *J* = 1.7 Hz, 3H), 3.00 (dd, *J*

= 6.7, 3.3 Hz, 4H), 2.43 (t, *J* = 4.9 Hz, 4H), 2.29 (s, 2H), 1.62 (td, *J* = 17.2, 15.8, 8.3 Hz, 2H), 1.44 (q, *J* = 7.2 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.1, 148.4, 144.2, 130.2, 121.3, 117.5, 117.4, 115.8, 115.5, 108.5, 107.4, 95.0, 57.7, 53.1, 49.4, 40.6, 27.2, 26.2, 23.9. MS (ESI$^+$) *m/z* 399 $[M+H]^+$.

6-amino-3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)benzo[*d*]thiazol-2(3*H*)-one (4d, WA416)

A solution of **3d** (1.0 g, 2.32 mmol) in 200 mL of methanol was hydrogenated over 10% Pd/C (0.30 g) under pressure (50 psi) for 2 h. The mixture was filtered through celite and evaporated under vacuum and the residue was purified by column chromatography (EtOAc:Hexane 1:1) to afford **4d** as brown residue (0.70 g, 75%). ^{1}H NMR (400 MHz, MeOD) δ 7.00 – 6.76 (m, 6H), 6.76 – 6.65 (m, 1H), 4.70 (s, 2H), 3.87 (q, *J* = 8.9, 8.0 Hz, 2H), 3.01 (dd, *J* = 6.5, 3.6 Hz, 4H), 2.48 (q, *J* = 10.1, 7.6 Hz, 4H), 2.33 (q, *J* = 7.8 Hz, 2H), 1.67 (hept, *J* = 8.6, 7.9 Hz, 2H), 1.53 (ddd, *J* = 15.1, 8.8, 6.0 Hz, 2H). ^{13}C NMR (101 MHz, MeOD) δ 169.8, 157.2 (d, *J* = 232.3 Hz), 148.0, 147.9, 144.3, 128.7, 123.2, 177.7 (d, *J* = 10.1 Hz), 115.0 (d, *J* = 20.2 Hz), 114.0, 111.7, 108.5, 57.4, 52.8, 49.5, 42.1, 25.2, 23.2. MS (ESI$^+$) *m/z* 402 $[M+H]^+$.

3-(4-(4-(4-Fluorophenyl)piperazin-1-yl)butyl)-6-isothiocyanatobenzo[*d*]oxazol-2(3*H*)-one dioxalate (5b, CM572)

Thiophosgene (0.03g, 0.29 mmol) was added dropwise to a solution of **4b** (0.10 g, 0.24 mmol) and triethylamine (0.11g, 1.07 mmol) in dry DCM at 0 °C under argon. After stirring the reaction mixture at this temperature for 2 h, the solvent was evaporated. The residue was purified by column chromatography (EtOAC) to afford **5b**, which was isolated as a dioxalate salt (white solid, 0.08 g, 53%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.79 (br s, 4H), 7.59 (s, 1H), 7.41-3.34 (m, 2H), 7.08 (t, *J* = 8.6 Hz, 2H), 7.01-6.99 (m, 2H), 3.86 (s, 2H), 3.31-3.08 (s, 10H), 1.72 (s, 4H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 163.4, 156.5 (d, *J* = 235.1 Hz), 153.7, 146.5, 141.9, 132.9, 130.7, 124.0, 122.3, 117.7 (d, *J* = 7.4 Hz), 115.4 (d, *J* = 21.8 Hz), 109.8, 108.1, 55.0, 50.8, 46.4, 41.4, 24.5, 20.6. HRMS calcd for $C_{22}H_{24}N_4O_2FS$ $[M+H]^+$ 427.1604, found 427.1601. Anal. calcd for $C_{26}H_{27}FN_4O_{10}S$: C, 51.48; H, 4.49; N, 9.24. Found: C, 51.94; H, 4.56; N, 9.44.

1-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-5-isothiocyanato-3-methyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one (5c, WA404)

To a solution of 1,1′-thiocarbonyldiimidazole (0.16 g, 0.91 mmol) dissolved in 2 mL of DMF was added dropwise a solution of **4c** (0.30 g, 0.75 mmol) and triethylamine (0.09 g, 0.91 mmol) in 3 mL of DMF, and the mixture was stirred for 5 min at 50 °C, then 30 min at room temperature. The reaction

mixture was then diluted with water and extracted with EtOAc. The combined organic layers were washed with water, brine, then dried over sodium sulfate, and filtered. The filtrate was concentrated under vacuum, and the residue was purified by column chromatography (EtOAc/Hexane 20:80) to afford **5c** as a brown residue (0.20 g, 60%). ^{1}H NMR (400 MHz, MeOD) δ 7.01 (tdt, *J* = 14.2, 9.6, 5.2 Hz, 2H), 6.90 (ddt, *J* = 16.2, 8.2, 4.9 Hz, 5H), 3.93 – 3.82 (m, 2H), 3.40 – 3.33 (m, 3H), 3.10 – 2.95 (m, 4H), 2.59 – 2.47 (m, 4H), 2.39 (dt, *J* = 16.8, 8.0 Hz, 2H), 1.78 (q, *J* = 8.2, 7.8 Hz, 2H), 1.58 (dd, *J* = 16.4, 8.9 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.4 (d, *J* = 232.3 Hz), 154.3, 148.4, 128.7, 117.5 (d, *J* = 10.1 Hz), 117.4, 116.8, 115.7, 115.6 (d, *J* = 20.2 Hz), 115.5, 107.1, 103.8, 57.7, 53.2, 49.4, 28.1, 27.3, 26.3, 23.9. MS (ESI$^+$) *m/z* 441 [M+H]$^+$.

3-(4-(4-(4-fluorophenyl)piperazin-1-yl)butyl)-5-isothiocyanatobenzo[*d*]thiazol-2(3*H*)-one (5d, WA435)

To a solution of 1,1′-thiocarbonyldiimidazole (0.11 g, 0.60 mmol) dissolved in 2 mL of DMF was added dropwise a solution of **4d** (0.20 g, 0.50 mmol) and triethylamine (0.03 g, 0.25 mmol) in 1 mL of DMF, and the mixture was stirred for 5 min at 50 °C, then 30 min at room temperature. The reaction mixture was diluted with water and extracted with EtOAc. The combined organic layers were washed with water, brine, then dried over sodium sulfate, and filtered. The filtrate was concentrated under vacuum, and the residue was purified by column chromatography (EtOAc/Hexane 20:80) to afford **5d** as a yellow oil (0.12 g, 54%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (d, *J* = 2.0 Hz, 1H), 7.17 (dd, *J* = 8.6, 2.1 Hz, 1H), 7.05 (d, *J* = 8.6 Hz, 1H), 6.95 (t, *J* = 8.7 Hz, 2H), 6.86 (dd, *J* = 9.1, 4.4 Hz, 2H), 3.97 (t, *J* = 7.3 Hz, 2H), 3.14 (q, *J* = 7.0, 4.8 Hz, 4H), 2.63 (t, *J* = 4.9 Hz, 4H), 2.49 (t, *J* = 7.3 Hz, 2H), 1.79 (p, *J* = 7.6 Hz, 2H), 1.65 (q, *J* = 7.5 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.3, 157.2 (d, *J* = 242.4 Hz), 147.8, 147.8, 136.3, 135.8, 126.5, 124.1, 119.9, 117.9 (d, *J* = 10.1 Hz), 115.5 (d, *J* = 20.2 Hz), 111.1, 57.4, 53.1, 50.0, 42.7, 25.3, 23.5. MS (ESI$^+$) *m/z* 443 [M+H]$^+$.

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