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To be cited as: *ChemMedChem* 10.1002/cmdc.201700302

Link to VoR: http://dx.doi.org/10.1002/cmdc.201700302

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chemmedchem.org

A NOVEL SMALL MOLECULE INHIBITOR OF PRION REPLICATION AND MUTANT PRION PROTEIN TOXICITY

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Keywords: Drug discovery, Neurotoxicity, Protein misfolding, Prion Diseases, Amino propargylic alcohols

ABSTRACT.

Prion diseases are a class of neurodegenerative disorders characterized by the accumulation in the brain of a self-replicating, misfolded isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}), a cell-surface glycoprotein of uncertain function. Emerging evidence suggests that PrP^{C} may passively serve as a substrate for the replication of PrP^{Sc}, and actively transduce its toxic effects to neuronal cells. The vast majority of previous drug screening campaigns have only targeted PrP^{Sc} replication, with largely unsuccessful results. We have previously described an experimental paradigm for detecting the cytotoxic effects of mutant PrP molecules in cultured cells, called drug-based cell assay (DBCA). Here, we employed this assay to screen a chemical library of diverse compounds, and identified a small molecule (molecule **55**) that counteracts the cytotoxic activity of a disease-associated point mutant of PrP. Importantly, this compound also blocks the replication of two different prion strains. Molecule **55** may represent the starting point for the development of a novel class of therapeutic agents for prion diseases.

MAIN TEXT.

Prion diseases are neurodegenerative conditions of human and animals characterized by dementia, motor abnormalities and cerebral amyloidosis. The main pathogenic event in these diseases is the accumulation in various brain areas of PrP^{Sc}, a misfolded and infectious isoform (prion) that propagates by imposing its abnormal conformation onto molecules of PrP^{C}, its endogenous counterpart.[1] Despite the fact that prion diseases have an annual incidence of only 1 to 2 cases per 1 million population, their scientific and medical relevance has grown enormously in the last two decades, for multiple reasons. In fact, human prion diseases can be transmissible, posing important medical concerns for organ transplants or blood transfusions.[2] Prions have also shown the ability to spread in domesticated as well as wild animals, and to cross species barriers, as in the case of the transmission of bovine spongiform encephalopathy to humans through contaminated food supplies.[3] Importantly, a prion-like mechanism has been proposed to explain the spread in brain tissues of different amylodogenic proteins associated with common neurodegenerative disorders, such as Alzheimer's or Parkinson's diseases, suggesting the alarming possibility that at least a subset of these disorders could be transmissible.[4] Unfortunately, despite more than three decades of intensive research, the molecular mechanisms laying at the root of prion infectivity and neurotoxicity are still poorly understood, and effective therapies for prion diseases are still missing.[5]

The formation, stability or metabolism of PrP^{Sc} have represented the main pharmacological targets of virtually all previous screening campaigns in prion diseases.[6] These approaches identified a number of compounds capable of blocking prion replication in cells, and occasionally delay the disease in mice. However, none of these molecules have shown efficacy in patients. Moreover, while multiple structural PrP^{Sc} conformers (called prion strains) may coexist in a host and possibly cause resistance to pharmacological treatments, the majority of anti-prion compounds identified so far act in a strain-specific fashion.[7] Collectively, these data challenge the concept that targeting exclusively PrP^{Sc} replication is a convenient therapeutic strategy in prion diseases. A possible complementary approach could be to also target neurotoxic pathways mediated by PrP^{C}.[8] We have previously described the

DBCA, a cellular assay based on the intrinsic cytotoxic effects of PrP molecules carrying mutations in the central region of the protein.[9] Multiple previous studies indicated that this assay is a useful tool to study PrP-mediated neurotoxic pathways, as well as to identify and characterize novel anti-prion compounds.[10] Here, we employed the DBCA to screen a representative diverse set of small molecules from an original collection of chemicals developed at EDASA Scientific.

The DBCA is based on the ability of PrP molecules carrying artificial deletions or naturally-occurring point mutations in the central region of the protein to highly increase the susceptibility of a variety of cultured cells and primary neurons to the toxicity of several cationic antibiotics, such as aminoglycosides (e.g. hygromycin or G418) or bleomycin analogues (e.g. Zeocin). Here, we employed this assay to screen a set of 54 molecules (Figures S1.A and S1.B) as representative heterobicyclic compounds available from the EDASA Scientific public compound library (www.edasascientific.com/page/catalogue). HEK293 cells stably expressing a PrP mutant carrying a 20 amino acids deletion in the central region (Δ105-125, called ΔCR) were exposed to Zeocin (500 µg/mL) for 48h, in presence (10 µM) or absence of each compound. Cell viability in the different conditions was then evaluated by MTT assay (Figure 1). As expected, cells exposed to Zeocin alone showed a strong reduction in viability (< 70%, not shown). Conversely, co-treatment with the porphyrin Fe(III)-TMPyP, previously shown to inhibit the activity of ΔCR PrP in the DBCA,[10] abrogated the effect (not shown). Among the different compounds, we identified eight molecules (**34**, **38**, **39, 43**, **46 , 50**, **53** and **54**) capable of rescuing at least 20% of antibiotic-induced cell death (Figure 2). We selected these compounds for subsequent analyses.

Previous evidence showed that compounds active in the DBCA could also possess the ability to inhibit prion replication in cell cultures.[11] Thus, we sought to test the potential anti-prion effects of our newly identified molecules. N2a cells chronically infected with the 22L mouse prion strain were exposed for 72h to Fe(III)-TMPyP (10 µM), vehicle control (DMSO, volume equivalent) or two different concentrations (10 and 30 µM) of each hit compound.

The levels of 22L prions were then evaluated by detecting the amount of proteinase-K (PK)-resistant PrP species by slot blotting (Figure 3). Consistent with previous reports, Fe(III)-TMPyP showed a potent (>80% inhibition) anti-prion effect. Among the eight different molecules identified by DBCA, five (**38**, **39**, **43**, **46** and **50**, Figure 3) showed an evident (>30%), statistically-significant inhibitory effect against 22L prion replication, at least at one concentration. These molecules represented the starting point for the assembly of an additional, focused collection of related compounds. For this purpose, we selected several EDASA compounds representing derivatives and/or having high (>80%) structural similarity with the eight molecules emerged in the primary screen. A second, focused library (compounds **55**-**98**, Figure 4.A and 4.B) exploring the chemical scaffold of each of the positive hits was built and subjected to activity validation by the different cellular assays.

The DBCA was used again to screen each compound of the new chemical library at three different concentrations (0.1, 1 and 10 μM) (Figure S2). We identified six compounds (**55**, **58**, **59**, **60**, **68** and **95**) capable of rescuing cell viability in the DBCA in a dose-dependent fashion (Figure 5). Interestingly, five of these molecules (**55**, **58**-**60** and **68**) share a high degree of chemical similarity, with a common alkynylcyclohexenylamine-based scaffold.[12] These compounds showed relatively low intrinsic cytotoxicity, as assayed in HEK293 expressing ΔCR or wild type (WT) PrP (Figure S3 and S4).

It is worth noting that the *N*-benzyl moiety was present in all the active compounds belonging to this subset, i.e. **55**, **58-60** and **68**. The key role of the *N*-benzyl moiety was further confirmed by the inactive analogues **57, 63, 64, 65, 66,** where this unit was missing. Interestingly compound **62**, which presents the *N*-benzyl moiety ramified with a methyl group, lost its activity as well. This ramification of the α carbon next to the amine was also present in the non-active compounds **63** and **64**. Among the active compounds, derivatives **58-60** presented an extra moiety on the amino group, that was an *N*-allylic moiety (**58** and **59**) or a *N,N*-dibenzyl moiety (**60**). The elongation of the acetylene unit by a phenyl ring seemed to be well tolerated in **55**, **59** and **60**. This observation may possibly lead to the conclusion that such position could represent an interesting point for adding chemical groups, with the objective of further exploring new potential anti-prions compounds.

In order the test the anti-prion activity of the six identified hits, we carried out a seven-point (0.03-10 μM) dose-response experiment in N2a cells infected with either the 22L or the RML prion strains. The levels of both prion strains were then evaluated by detecting the amount of proteinase-K (PK) resistant PrP species by Western blotting (Figure 6 and Figure S5 and S6). One compound (an amino propargylic alcohol derivative, called **55**) showed the ability to robustly block the replication of both 22L and RML prion strains, at concentrations in the low micromolar range. Collectively, these results indicated that compound **55** effectively silences the cytotoxic effect of ΔCR PrP, and inhibits prion replication in a strain-independent manner. Interestingly, a previous study reported only a partial, although still surprising pharmacological correlation between compounds active in the DBCA and against prion propagation in cell cultures.[11] Our data support a similar conclusion, since we identified compounds active only in the DBCA (58, 59, 60, 68 and 95), or in both assays (molecule 55). A possible, logic explanation for these observations is that the different pharmacological targets of the identified molecules may play roles in just mutant PrP-dependent toxicity, or also in prion replication.

In its standard format, the DBCA relies on the expression of ΔCR PrP. However, the deletion carried by this PrP mutant is artificial, and not associated with any human prion disease. In order to further validate the activity of molecule **55** in a more disease-relevant experimental context, we turned to another PrP mutant, carrying the mouse homologue (G113V) of a single point substitution (G114V) which in humans is associated with an early-onset form of Gerstmann-Straüssler-Scheinker syndrome (GSS). Consistent with previous reports, we found that the expression of G113V PrP in cultured cells confers hypersensitivity to cationic antibiotics in the DBCA similarly to ΔCR PrP, although with a lower efficiency (Figure 7). Importantly, co-treatment with molecule **55** (30 μM) significantly rescued the effects. These results demonstrate that molecule **55** is also capable of inhibiting the cytotoxic effect of a PrP mutant associated with an inherited prion disease.

In order to rule out the possibility that the rescuing effects shown by compound **55** in the different DBCA experiments were due to an interaction of the molecule with Zeocin, we performed the assay in two modified versions. In contrast to mutant PrP, expression of WT PrP does not confer

hypersensitivity to Zeocin.[9,10] Therefore, we performed the DBCA in HEK293 cells stably expressing WT PrP, with increasing concentrations of Zeocin (0-2.000 μg/mL), in presence or absence of molecule **55** (Figure S7). In cells not treated with **55**, we observed a dose-dependent toxicity of Zeocin over 72h, with approximately 50% of cell death obtained at the highest concentration (2.000 μg/mL). Importantly, we failed to observe any significant rescue upon incubation with molecule **55** (3-10 μM).

Next, we used HEK293 cells stably expressing ΔCR PrP, but replaced Zeocin with G418, an aminoglycoside previously shown to produce similar mutant PrP-dependent effects in the DBCA. Consistent with the previous data, molecule 55 induced a robust, dose-dependent rescue of cell viability (Figure S9). Collectively, these results clearly indicate that the rescuing effects of compound 55 in the DBCA are antibiotic-independent, and specific for mutant PrP expression.

One possible mechanism by which molecule **55** may exert its different anti-prion effects is by targeting directly or indirectly PrP^C. We tested this possibility in three different ways. First, we checked whether the compound suppresses the expression of PrP^C. HEK293 cells stably expressing ΔCR PrP, or N2a cells stably expressing WT PrP, were treated with raising concentrations of molecule **55**, the anti-PrP porphyrin TMPyP (10 μM), or vehicle control (volume-equivalent), and PrP expression was evaluated by Western blotting (Figure 8). We found no difference in the levels of PrP^C between treated vs untreated cells.

Next, we tested whether molecule **55** acts by promoting the removal of PrP^C from the cell surface. In this case, we analyzed the distribution of an EGFP-tagged WT PrP stably expressed in HEK293 cells, in presence or absence of different concentrations of molecule **55** (Figure 9). As a positive control, we used chlorpromazine (CPZ), an antipsychotic known to inhibit clathrin-mediated endocytosis (CME), and recently found to cause the redistribution of PrP^C from the plasma membrane (manuscript under review). Even in this case, we found no evidence that molecule **55** alters the cell surface distribution of PrP^C.

Finally, we directly tested the possibility that molecule **55** may act by directly binding to PrP^C, by using dynamic mass redistribution (DMR), a biophysical technique previously employed to test the

interaction of small ligands to PrP^C.[10] As a positive control for the technique, we confirmed the interaction of TMPyP to PrP^C, as previously reported (Figure 10). Conversely, we failed to detect a specific interaction of molecule **55** to PrP^C. These data were confirmed using surface plasmon resonance (SPR), another biophysical technique previously umployed to detect the interaction of small molecules to PrP^C in a kinetic fashion.[10] Collectively, these results indicate that **55** does not exert its anti-prion effects by targeting PrP^C, but rather through an unknown pharmacological target, which could play a role in both mutant PrP toxicity and prion replication.

On the basis of the promising results described in this manuscript, we calculated key descriptors (Table 1 and Supporting Information) to evaluate drug-likeness for compound **55**, and thus its potential as a therapeutic agent for the central nervous system (CNS). Indeed, in the last few years compounds active on the CNS have extensively been characterized. As a result, sets of physicochemical and pharmacokinetic parameters that may be used for the identification of successful CNS molecules have been proposed (examples are reported in Table 1).[13] These analyses suggest that compound **55** might possess a desirable drug-like profile for oral dosage and for targeting the CNS, as all calculated properties are in line with the recommended guidelines for CNS-directed therapeutics.[14,15]

In summary, we have used the DBCA assay, a previously described method to detect the cytotoxic activity of PrP mutants, to sequentially screen a highly diverse set of original compounds from the EDASA Scientific public compound library, and a second more focused subset of molecules built around the scaffold of the positive hits arising from the primary screen. This approach identified compound **55**, an amino propargylic alcohol derivative,[16] as capable of inhibiting the cytotoxic effect of a PrP mutant carrying a deletion in central region (residues 105-125, called ΔCR PrP), as well as of the mouse homologue of a human disease-associated point mutant (G114V). Importantly, the molecule also blocked the replication of two different prion strains in neuroblastoma cells. These results indicate that molecule **55** possesses the remarkable ability of acting against both prion replication and mutant PrP toxicity. However, the compound does not seem to target PrP^C, either directly or indirectly, suggesting the intriguing possibility that its pharmacological target could be a

molecular factor playing a role in both prion replication and mutant PrP toxicity. Thus, in addition to provide a starting point for the development of a novel class of therapeutic agents for prion diseases, molecule **55** could represent an important tool to gain insights into the mechanisms underlying prion replication and toxicity.

FIGURE LEGENDS.

Figure 1. The DBCA was employed to evaluate the anti-ΔCR PrP effects of each individual molecule. The bar graph illustrates the quantification of the rescuing ability of each molecule. Mean values were obtained from two independent experiments, and expressed as percentage of cell viability rescue. Compounds **27, 35, 40, 41, 42, 44, 52** were found to be insoluble at the lowest concentration used in the assay, and thus were not analyzed.

Figure 2. The chemical structure of the positive hits from the primary DBCA-based screen are shown. Numbers indicate (from left): (i) Sequential numbering (**1** to **54**); (ii) EDASA chemical library code; (iii) Code of the *in house* chemical library of the Dulbecco Telethon Laboratory of Prions & Amyloids at University of Trento.

Figure 3. Positive hits from the primary screen are validated by prion replication assay in 22L-infected cells. N2a cells chronically infected with the 22L prion strain were incubated with two different concentrations (10 and 30 μM) of each compound for 72h. The level of PK-resistant PrP before and after treatment was estimated by slot blotting, using anti-PrP^C antibody 6D11. Signals were quantified by densitometric analysis of blots, normalized on the total amount of proteins (obtained by Ponceau staining) in PK-untreated replicates, and expressed in the bar graph as mean percentage (%) of the signal in vehicle-treated cells (± standard error). Statistical differences between each molecule/concentration and vehicle control were estimated by Student *t*-test (**p* < 0.05; **p* < 0.01).

Figure 4.A. **Chemical structures of molecules 55-67.** These molecules were selected from the EDASA Scientific public compound library for their similarity with compound **50**.

Figure 4.B. **Chemical structures of molecules 69-98.** These molecules were selected from the EDASA Scientific public compound library for their similarity with compound **38, 39, 43** and **46**.

Figure 5. Hits from the focused library were tested by DBCA. Graph shows mean values (± standard error) expressed as percentage of cell viability rescue using the equation: R = (T-Z)/(U-Z) (R: rescuing effect; T: cell viability in compound-treated samples; Z: cell viability in zeocin-treated samples; U: cell viability in untreated samples). Data were obtained from a minimum of 4 independent experiments (n ≥ 4).

Figure 6. Compound **55** showed the ability to inhibit the replication of both RML (picture in panel A, quantification in panel C) and 22L (B) prion strains. The level of PK-resistant PrP before and after treatment was estimated by western blotting, using anti-PrP^C antibody D18. Signals were quantified by densitometric analysis of images, normalized on the total amount of proteins (obtained by Ponceau staining) in PK-untreated replicates, and plotted in the graphs as mean percentage (%) of the signal in vehicle-treated cells (± standard error). Inhibitory concentrations at 50% (IC_{50}) values for molecule 55 were: 8.25 µM against the 22L strain, and 3.21 µM against the RML strain.

Figure 7. Molecule **55** was tested in the DBCA against a disease-associated PrP mutant. The DBCA was performed as before, with slight modification (see Supplementary Information). Mean values were obtained from 8 independent experiments (n = 8), and expressed as percentage of cell viability in vehicle (VHC)-treated control.

Figure 8. Western blotting analyses indicate that molecule **55** does not reduce the expression of PrP. HEK293 cells expressing ΔCR PrP or N2a cells expressing WT PrP were treated with the indicated concentrations of molecule **55** for 48h. PrP signals were detected by anti-PrP antibody D18. The porphyrin TMPyP (TP) and vehicle (DMSO) were used as negative controls. The experiment was repeated three independent times (n = 3).

Figure 9. Treatment with molecule **55** did not induce the relocalization of EGFP-PrP from the cell surface. HEK293 cells stably expressing EGFP-PrP were grown to ~60% confluence on glass coverslips, and then treated with the indicated concentrations of molecule **55** or CPZ for 48h. After

fixation and washing, the intrinsic green signal of EGFP-PrP was acquired. In contrast to molecule **55**, CPZ produced EGFP-PrP relocalization from the cell surface.

Figure 10. DMR analyses indicate that molecule **55** does not bind to recombinant PrP^C. Different concentrations (0.03-100 μM) of molecule **55** or TMPyP were added to label-free microplate well surfaces (EnSpire-LFB HS microplate, Perkin Elmer) on which full-length mouse recombinant PrP^C had previously been immobilized. Measurements were performed before (baseline) and after (final) adding the compound. The response (pm) was obtained subtracting the baseline output to the final output signals. The output signal for each well was obtained by subtracting the signal of the protein-coated reference area to the signal of uncoated area. The data for TMPyP (black dots) were fitted (blue line) to a sigmoidal function using a 4 parameter logistic (4PL) non-linear regression model (R^2 = 0.96).

Table 1.[a,b] First value indicates the median value derived for a set of 119 marked CNS drugs [13c] and a set of 108 Pfizer CNS clinical candidates, [13c] respectively; numbers in brackets is the range values for the same set. [c] Suggested values for increasing the potential for blood–brain barrier (BBB) penetration and oral bioavailability.[13a,b] [d] Calculated octanol/water partition coefficient. [e] Calculated distribution coefficient. [f] Molecular weight. [g] Topological polar surface area. [h] Number of hydrogen bond donors. [l] Most basic center. [l] Intrinsic aqueous solubility.[m] Classification for human intestinal absorption; [n] Classification of BBB penetration. [o] Predicted BBB penetration value. [p] Classification of P-glycoprotein transport.

EXPERIMENTAL SECTION

See Supporting Information for experimental details.

ACKNOWLEDGEMENTS.

The authors thank Alessandro Negro (University of Padova) for providing recombinant mouse PrP^C, Roberto Chiesa for the mouse prion inocula (RML and 22L) and BioSolveIT GmbH (http://www.biosolveit.de) for providing academic license of SeeSAR program. The study was supported by a Young Investigator Award from the Italian Ministry of Health to EB and MLB, by a grant from the Russian Foundation for Basic Research (RFBR; Projects No. 14-03-01114), and a grant from the CJD Foundation to EB (GR-2010-2312769). SRE was supported by a grant from the E-Rare Joint Transnational Call (E-Rare-2). EB is an Assistant Telethon Scientist at the Dulbecco Telethon Institute (TCP14009, Fondazione Telethon, Italy).

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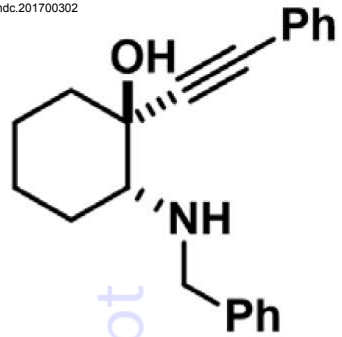
ChemMedChem

10.1002/cmdc.201700302

Prion diseases are neurodegenerative disorders characterized by the accumulation in the brain of a self-replicating, misfolded isoform (PrP^{Sc}) of the cellular prion protein (PrP^{C}). No therapies are available for these pathologies. We have capitalized on previously described cell-based assays to screen a library of small molecules, and identified **55**, a compound capable of counteracting both prion replication and toxicity. Molecule **55** may represent the starting point for the development of a completely novel class of therapeutics for prion diseases.



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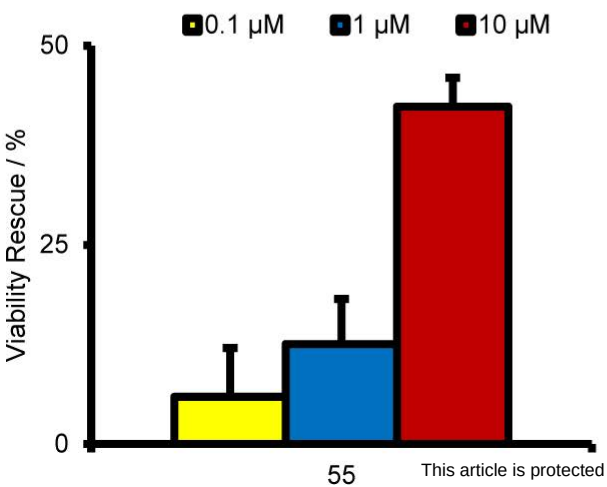

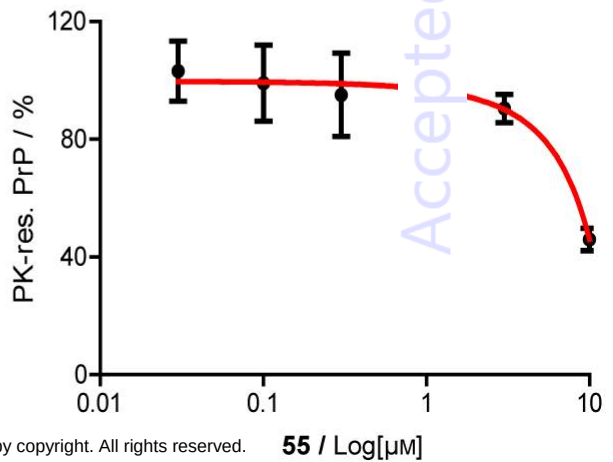

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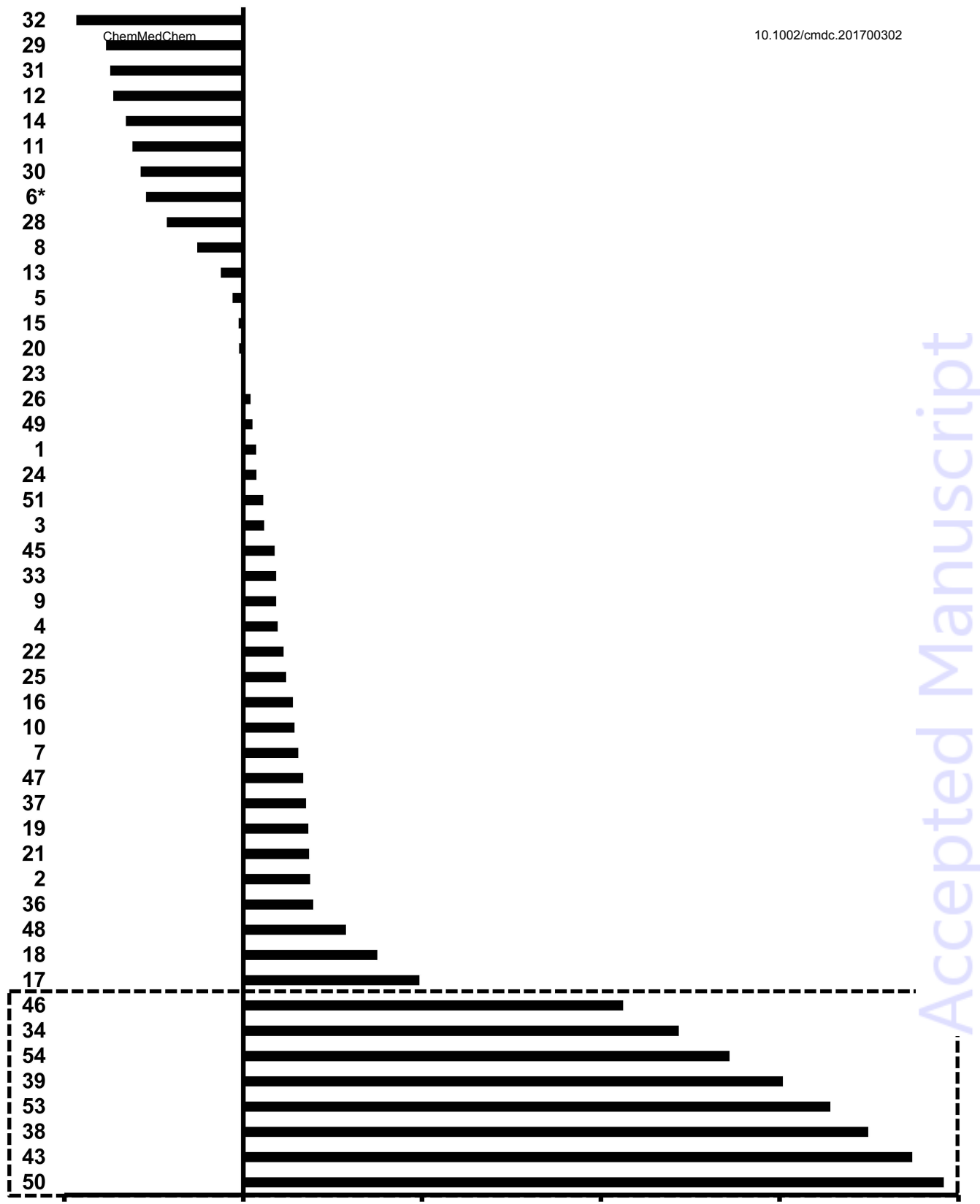

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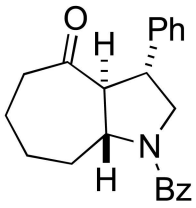

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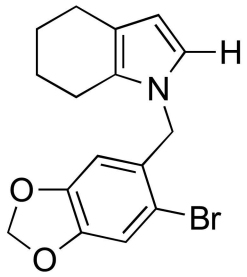

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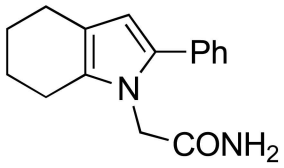

39, BB 0266682
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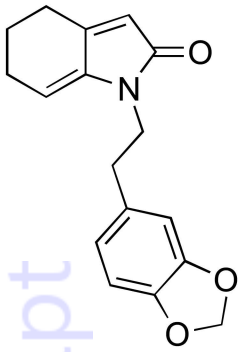

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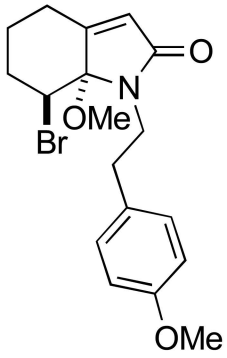

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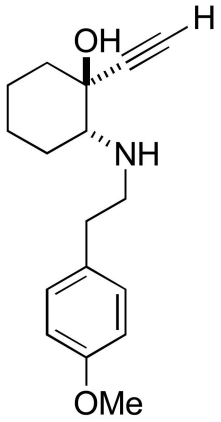

50, BB 0300691
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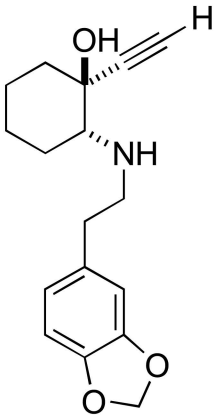

53, BB 0300695
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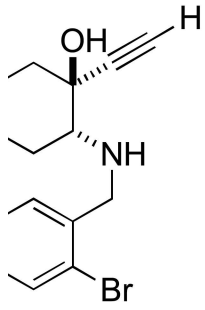

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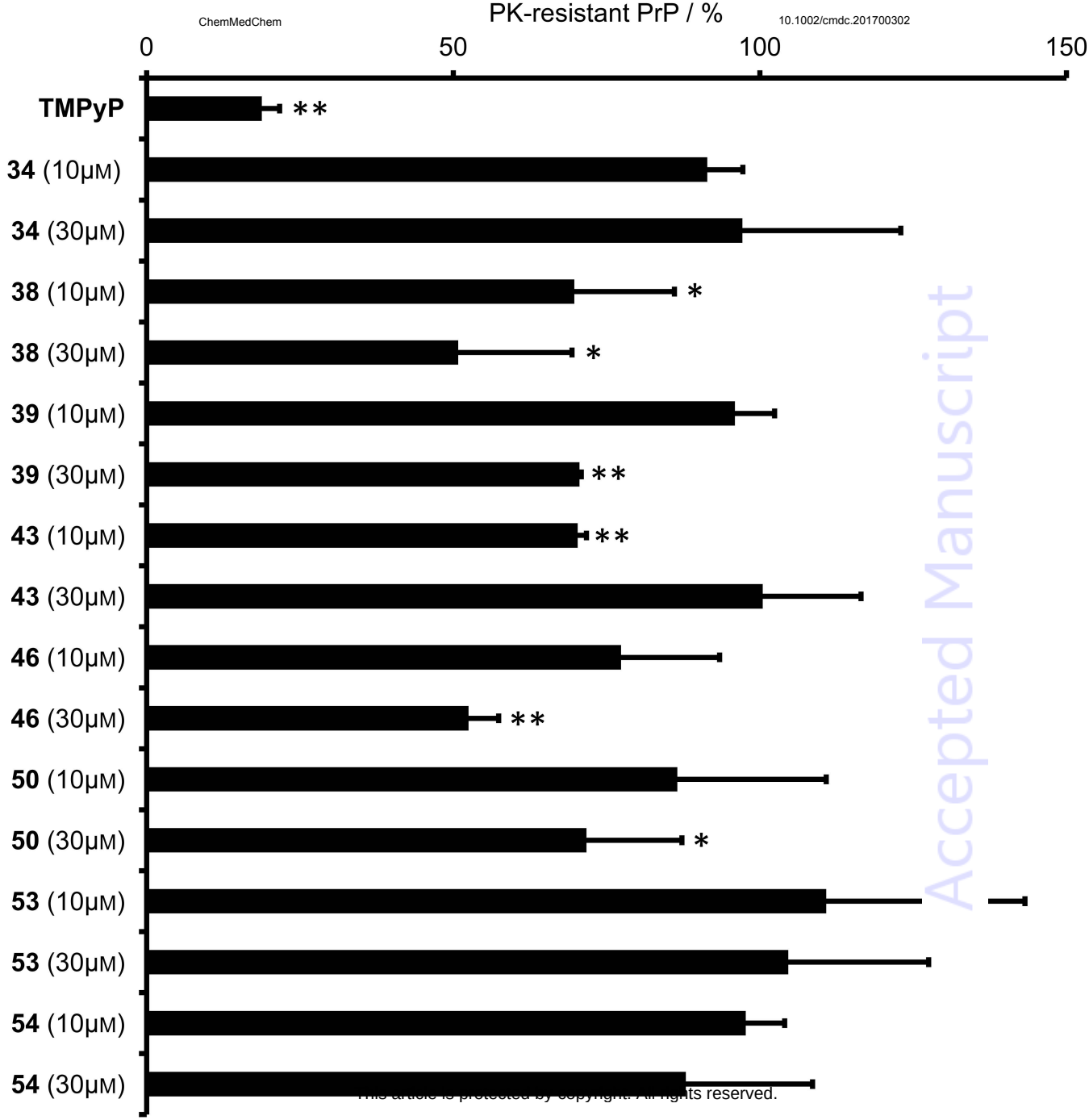
10.1002/cmdc.201700302



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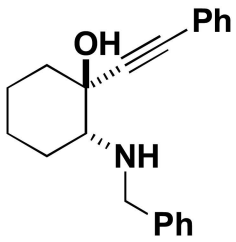

55, BB 0300674
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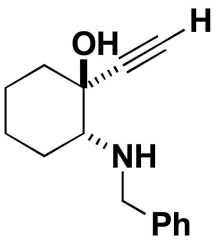

56, BB 0300676
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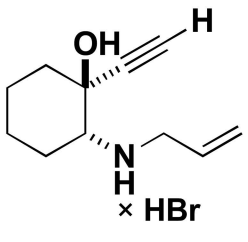

57, BB 0300677
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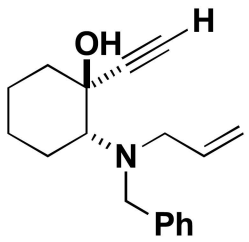

58, BB 0300666
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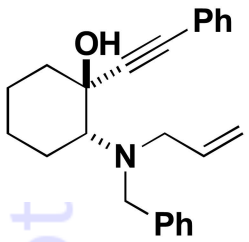

3 0300657
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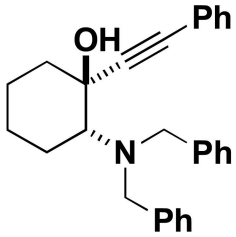

60, BB 0300647
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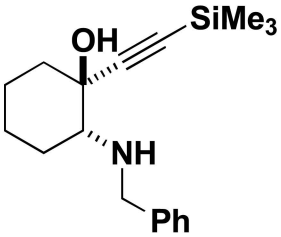

61, BB 0300658
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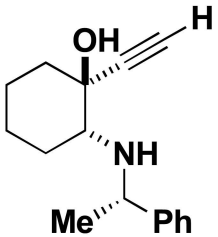

62, BB 0304311
SM560

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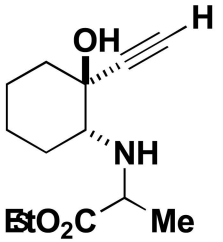

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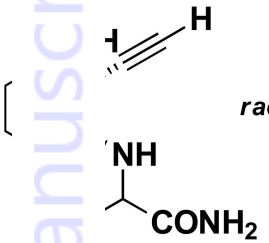
SM554

rac



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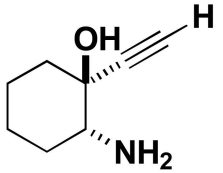
0300648



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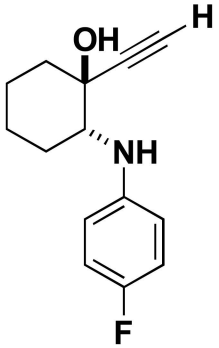

66, BB 0300660
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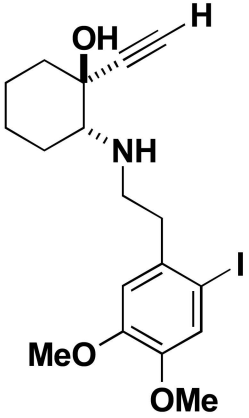

67, BB 0300654
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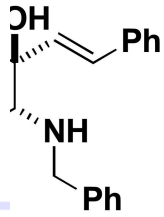

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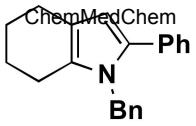
SM561

10.1002/cmdc.201700302



69, BB 0267327
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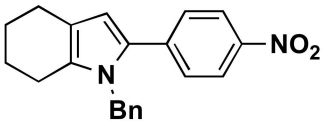

70, BB 0267329
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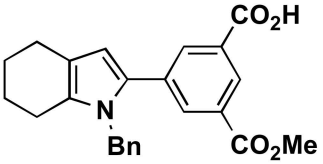

71, BB 0266677
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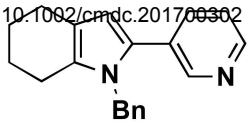

72, BB 0266680
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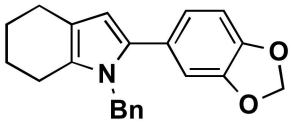
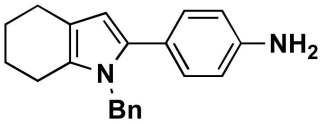


73, BB 0267338
SM528

74, BB 0267340
SM530



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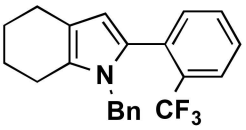
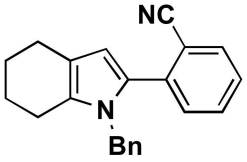


76, BB 0267366
SM533



77, BB 0267368
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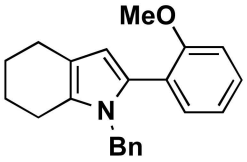

78, BB 0267387
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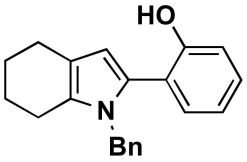

79, BB 0267388
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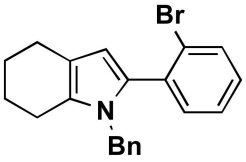

80, BB
0267328
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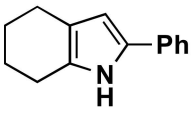
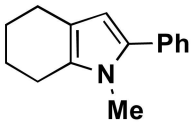


81, BB 0267339
SM529



82, BB 0269301
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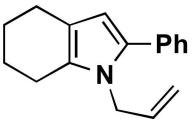

83, BB 0267332
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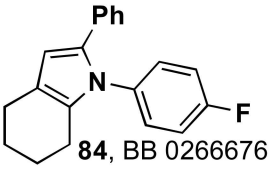

84, BB 0266676
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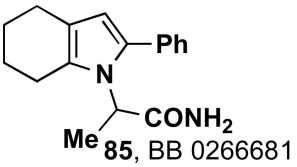

85, BB 0266681
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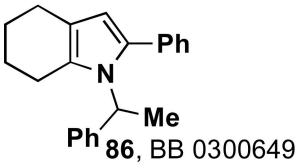

86, BB 0300649
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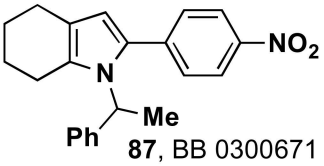

87, BB 0300671
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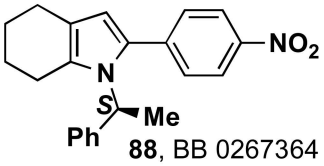

88, BB 0267364
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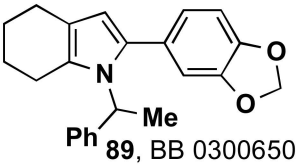

89, BB 0300650
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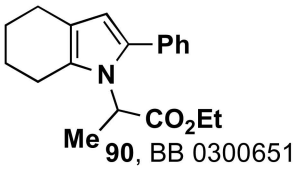

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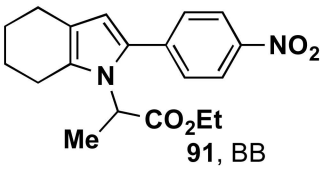
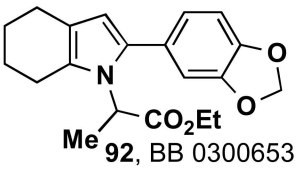


91, BB
0300652
SM537



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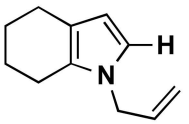

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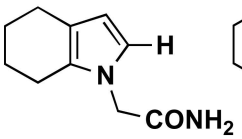

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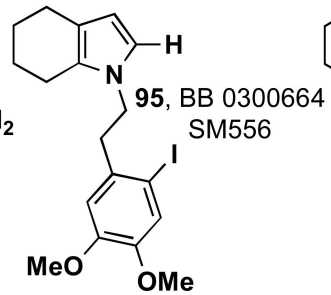

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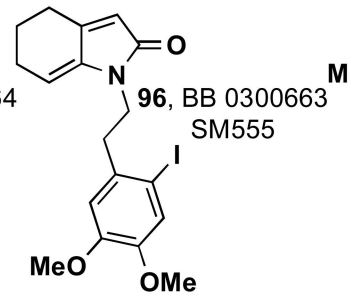

96, BB 0300663
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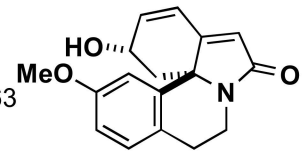

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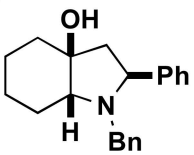

98, BB 0268163
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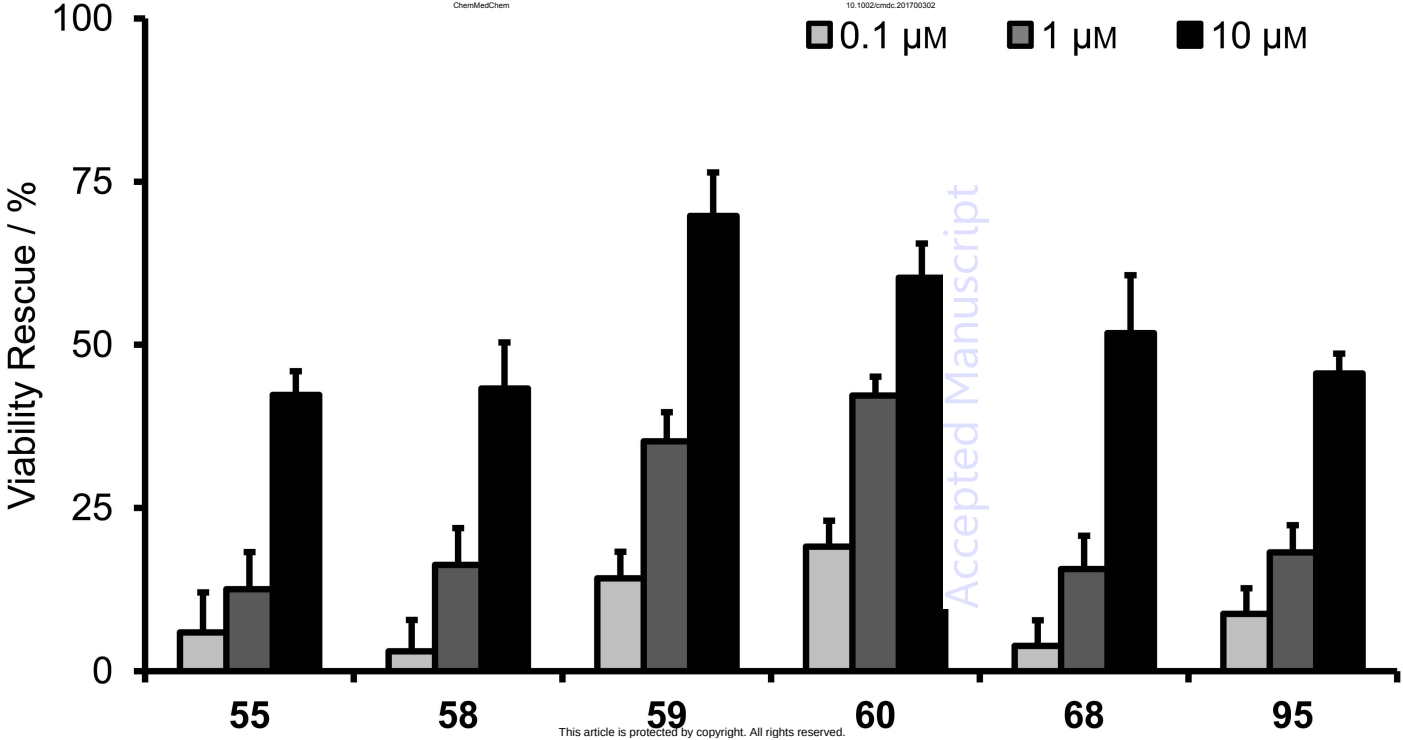

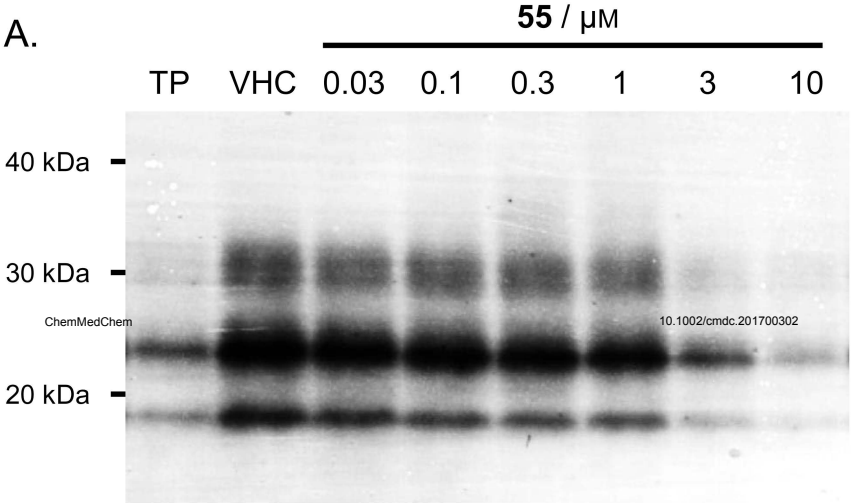

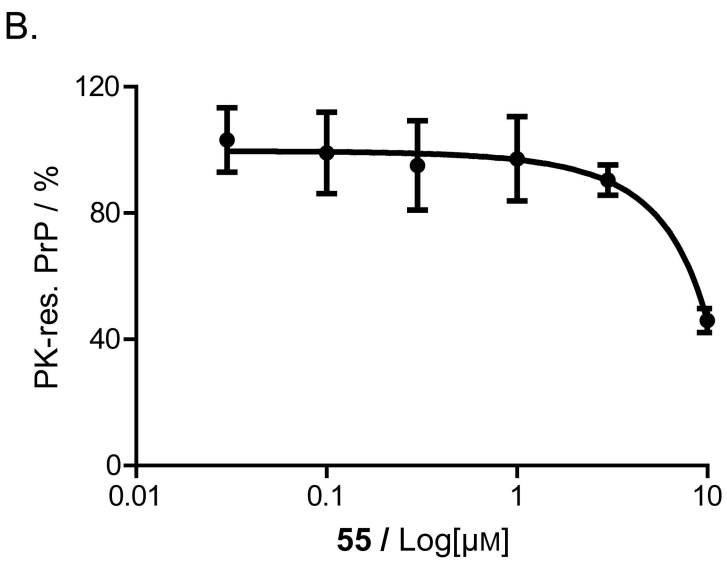

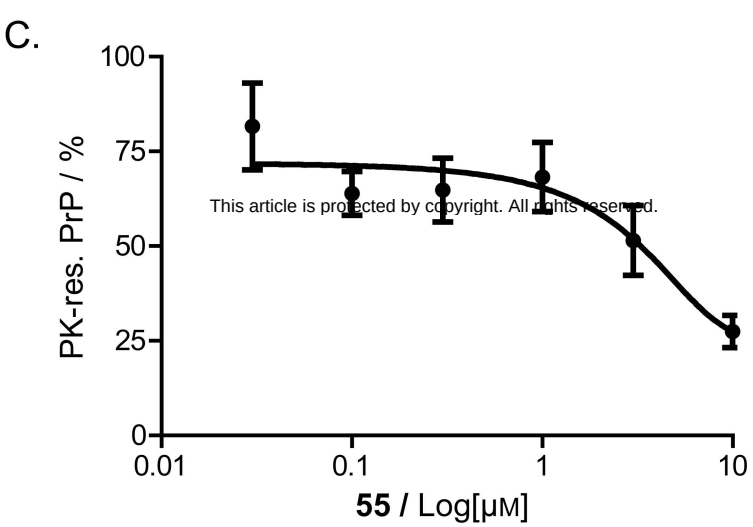

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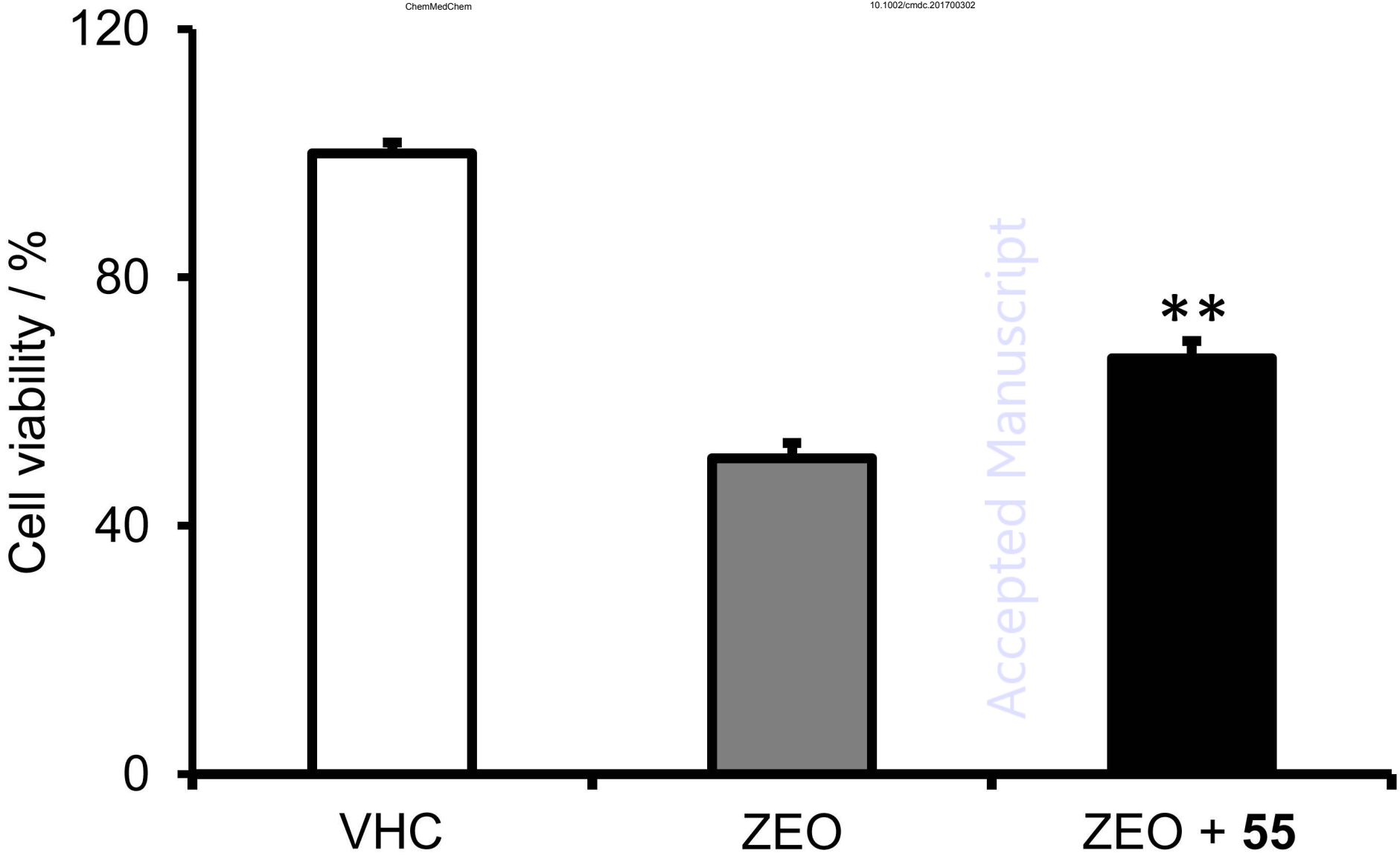

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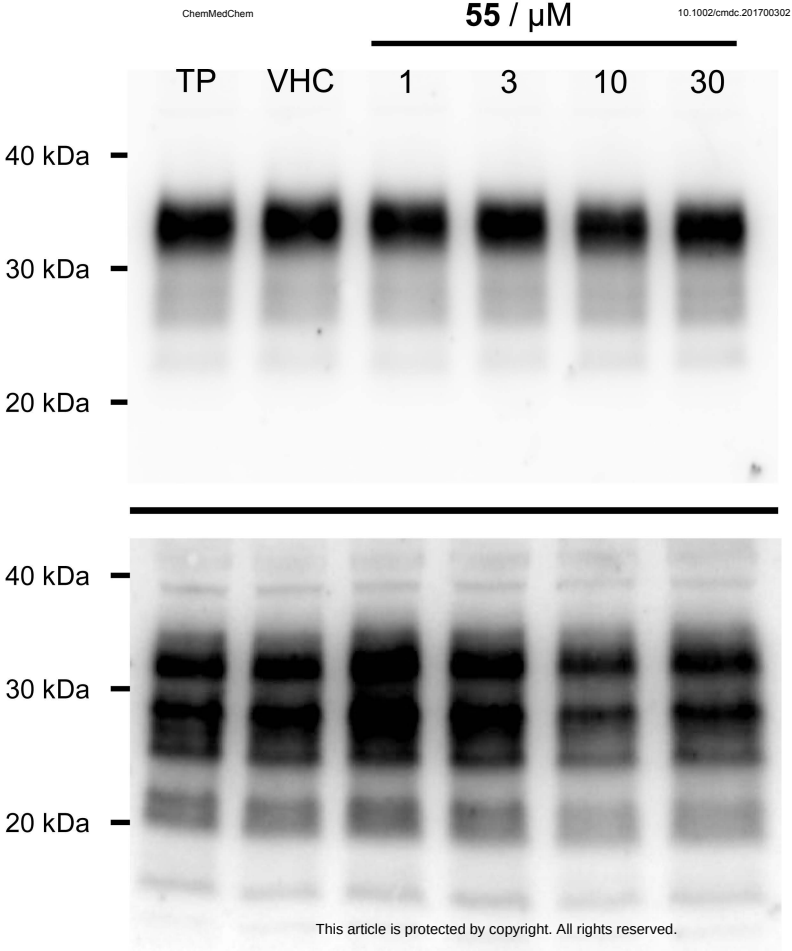

EK293

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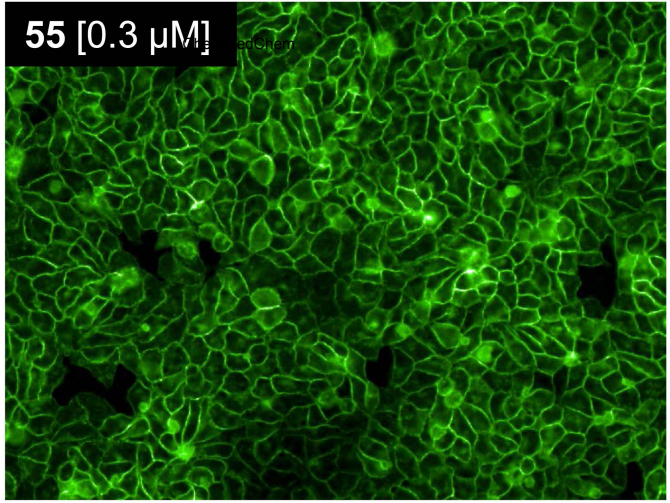

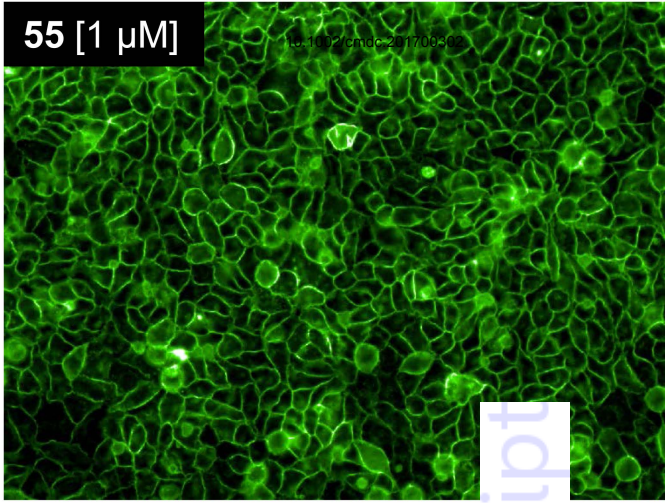

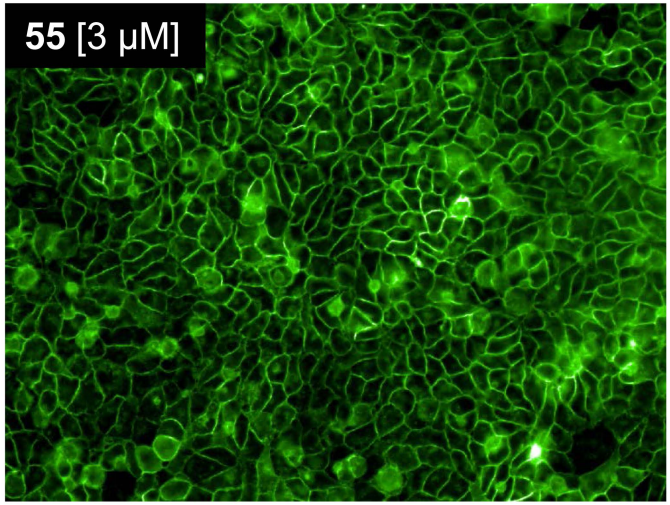

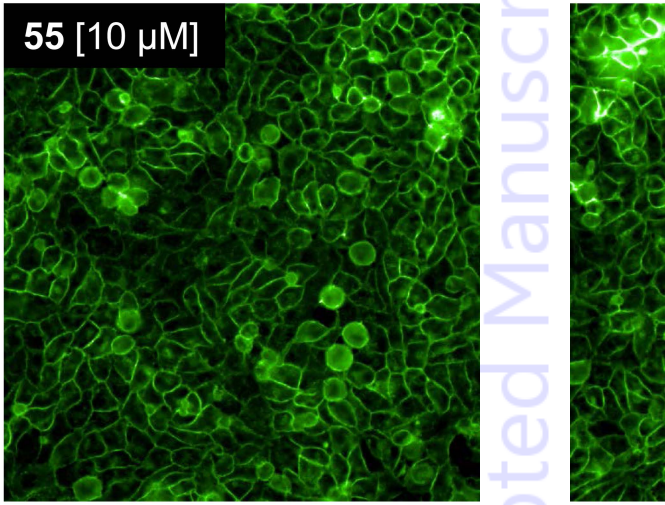

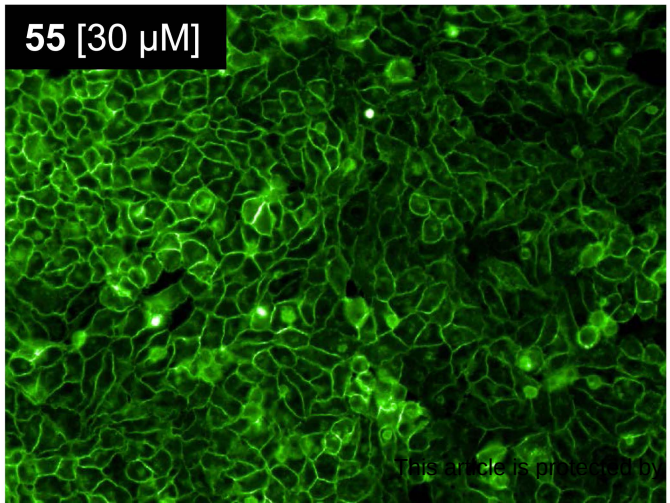

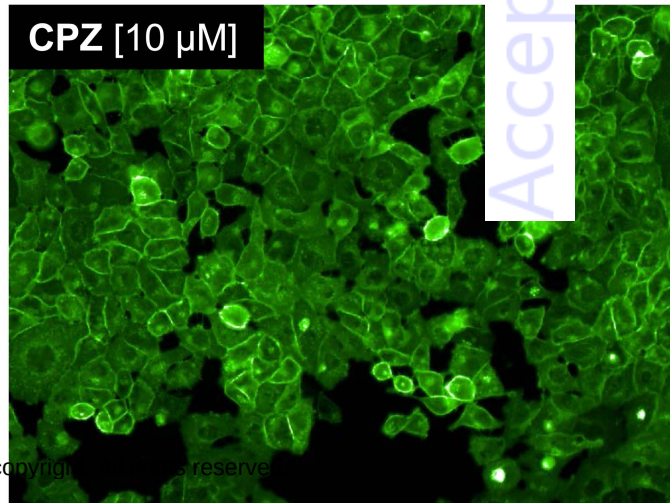

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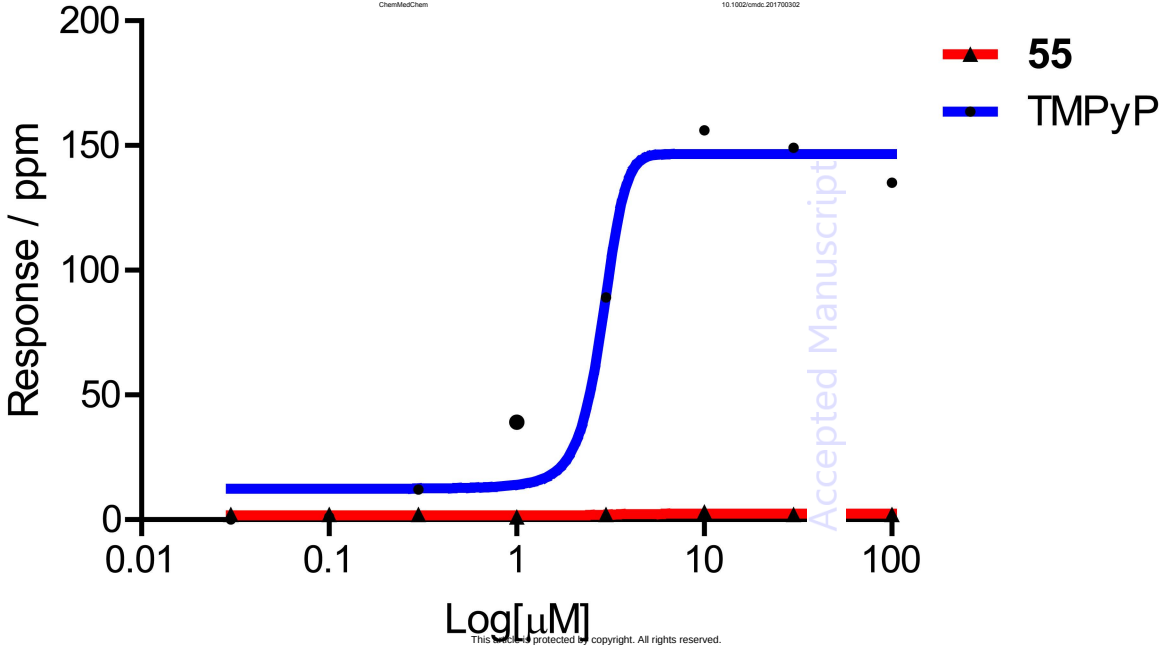

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Table 1. Property guidelines for CNS druglike candidates

	Drugs[a]	Clinical candidates[b]	Literature Review[c]	55
logP[d]	2.8 (0.4-5.1)	3.31(0.2-5.8)	2-5	3.5
logD[e]	1.7 (-0.5-3.8)	2.2 (-0.6-4.9	2-5	1.6
MW[f]	305.3 (181.2-426.5)	360.4 (252.5-46 0))	< 500	305.4
tPSA (A^2)[g]	44.8 (16.1-86.2)	51.2 (21.3-80.( )	< 90	32.36
HBD[h]	1 (0-3)	1 (0-3)	<3	2
pKa[i]	8.4 (3.9-10.1)	8.4 (5.7-10.3		7.8
logS[l]			> 1	1.732
HIA[m]			+	+
BBB category[n]			+	+
logBB[o]			-0.2-1	0.37
P-gp category[p]			no	no