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Structure-based lead optimization to improve antiviral potency and ADMET properties of phenyl-1H-pyrrole-carboxamide entry inhibitors targeted to HIV-1 gp120

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Abstract

We are continuing our concerted effort to optimize our first lead entry antagonist, NBD-11021, which targets the Phe43 cavity of the HIV-1 envelope glycoprotein gp120, to improve antiviral potency and ADMET properties. In this report, we present a structure-based approach that helped us to generate working hypotheses to modify further a recently reported advanced lead entry antagonist, NBD-14107, which showed significant improvement in antiviral potency when tested in a single-cycle assay against a large panel of Env-pseudotyped viruses. We report here the synthesis of twenty-nine new compounds and evaluation of their antiviral activity in a single-cycle and multi-cycle assay to derive a comprehensive structure-activity relationship (SAR). We have selected three inhibitors with the high selectivity index for testing against a large panel of 55 Env-pseudotyped viruses representing a diverse set of clinical isolates of different subtypes. The antiviral activity of one of these potent inhibitors, **55** (NBD-14189), against some clinical isolates was as low as 63 nM. We determined the sensitivity of CD4-binding site mutated-pseudoviruses to these inhibitors to confirm that they target HIV-1 gp120. Furthermore, we assessed their ADMET properties and compared them to the clinical candidate attachment inhibitor, BMS-626529. The ADMET data indicate that some of these new inhibitors have comparable ADMET properties to BMS-626529 and can be optimized further to potential clinical candidates.

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Graphical Abstract

Keywords

HIV-1; ENV-pseudovirus; virus entry antagonist; broad spectrum; structure-activity relationship (SAR); “CH₂OH” switch hypothesis; ADMET

1. Introduction

Advances in available therapeutics, in particular combination antiretroviral therapy (cART), significantly improved the treatment of HIV infection and facilitated the shift from high morbidity and mortality to a manageable chronic disease. Despite this remarkable success, current therapies suffer from several limitations. For example (1) reliance on daily adherence; (2) long-term use resulting in long-term toxicity; (3) limited treatment options due to the development of drug resistance; (4) high cost, and finally, (5) the non-curative nature of current treatments. Further, despite tremendous effort and investment, an effective vaccine or microbicide is not yet available, and significant hurdles must be overcome to achieve a functional cure. Thus, the continued development of small-molecule drugs with high potency against novel targets but minimal side effects is imperative. The development of novel therapeutics will aid in increasing the number of new drugs available and will extend the scope of combination therapy. Our group has made significant headway in filling this critical need by developing a new class of HIV-1 entry inhibitors targeted to the Phe43 cavity of HIV-1 gp120, which is distinct from the binding site of the clinical candidate attachment inhibitor from BMS [1].

Viral attachment and fusion to the host cell membrane are critical for HIV-1 to enter host cells and initiate its life-cycle by utilizing host cell machinery [2]. Therefore, viral attachment and fusion (often collectively termed “viral entry”) have been targets of new drug discovery for many years [3–6]. There are only two drugs currently on the market that target the entry pathway. FUZEON® (enfuvirtide), a peptide-based drug, targets the envelope glycoprotein gp41 [7, 8] and SELZENTRY® (maraviroc), a small molecule drug, targets the host cell receptor CCR5 [9, 10].

Despite the fact that HIV-1 gp120 is critical for viral entry and has been the target of prior drug discovery efforts, no drugs that target gp120 have been approved yet by the US FDA. BMS-663068, the most advanced inhibitor in this class, is a prodrug of BMS-626529. The safety and efficacy of BMS-663068 were demonstrated recently in a Phase 2b clinical trial, and the compound is currently undergoing Phase III clinical trials. In 2015, BMS-663068 received the “*Breakthrough Therapy Designation*” from the US FDA, indicating the importance of gp120 as a target for the development of drugs that prevent viral entry into

host cells. This recognition by the FDA confirms that our efforts to develop novel drugs are highly significant, especially for the growing number of treatment-experienced patients with limited treatment options and for use as a combination therapy for the millions of affected individuals worldwide.

Our laboratory had focused on developing novel inhibitors targeted to the Phe43 cavity of gp120 since 2005 when we first reported the discovery of NBD-556, the NBD-series CD4-mimic [11]. Unfortunately, NBD-556 enhanced HIV-1 infection in CD4⁺-CCR5⁺ cells and thus behaved as an HIV-1 entry agonist [12, 13]. Since this finding, we and others have endeavored to design CD4 mimics with higher potency, lower toxicity, and devoid of this undesirable agonist property [13–21]. However, progress was slow until we determined the crystal structure of HIV-1 gp120 in complex with NBD-556 [22], which guided us the modification of NBD-556 to NBD-11021, an entry antagonist that is a more potent entry inhibitor [15, 23]. Since then, we determined the X-ray structures of several new generations of the NBD series HIV-1 entry antagonists in complex with gp120 [13, 14, 22, 24, 25]. The structural knowledge provided valuable information for the design of our next generation of inhibitors, which achieved measurable improvements in both potency and selectivity index (SI) against a large panel of >50 Env-pseudotyped viruses representing a diverse set of clinical isolates of different subtypes. Our first entry antagonist NBD-11021, which exhibited ~3-fold higher antiviral activity and ~1.3-fold higher SI compared to the entry agonist, NBD-556. Hence, we optimized our first candidate antagonist lead NBD-11021 and developed several next-generation leads to higher activity and SI (Figure 1) [13–15, 24].

In this report, we describe our concerted effort in optimizing the best lead compounds so far using a structure-based drug design approach. This effort resulted in a new HIV-1 entry antagonist, **55**, which exhibited an extraordinary broad-spectrum activity against a large panel of Tier II HIV-1 clinical isolates and 2 Tier III HIV-1 clinical isolates (NIH #11022 and 11605). The antiviral activity of **55** against some clinical isolates was as low as 63 nM. A comparison of the *in vitro* ADMET data from the best NBD series inhibitors with the attachment inhibitor BMS-626529 indicated that **55** has desirable ADMET profiles as a promising lead for further development [24].

2. Results and Discussion

2.1. Structure-based lead optimization

Recently, we reported the crystal structure of HIV-1 gp120 in complex with NBD-14010, one of the new series of molecules, at a resolution of 2.1-Å (PDB ID: 5U6E) [24]. This structure provided the critical information that the methyl group in the thiazole ring does not contribute to any interactions with gp120; instead, it is located in the solvent-exposed region outside the Phe43 cavity. Furthermore, we also observed that the CH₂OH group does not interact with gp120 (Figure 2a). Based on these observations, we *hypothesized* the following:

1. Removal of the solvent-exposed CH₃ from the thiazole ring will induce favorable interactions of the molecules with gp120.

2. Positional switching of the CH₂OH group from C5 to C4 of the thiazole ring will lead to enhanced interactions with gp120.

Based on our hypotheses, we previously removed the CH₃ from NBD-14010 to create NBD-14107, which displayed excellent breadth in anti-HIV-1 activity [24]. However, the improvement was not significantly higher than NBD-14010 [16]. Therefore, for this study, we decided to move CH₂OH group from C5 to C4 of the thiazole ring to generate a model compound. A Glide-based (Schrodinger, LLC) docking simulation of this model compound revealed that the top-scoring compound (XP score = -10.902 Kcal/mol) was the one that we designed based on our second hypothesis. The CH₂OH formed an H-bond with Met426 (Figure 2b), which was recognized as a critical residue for the binding of the CD4 Phe43 [26]. The overlay structures of NBD-14010 and the modeled structure in 3D in Figure 2c show that the CH₂OH and CH₃ of NBD-14010 made no interaction with gp120. A recent report on the coevolution analysis of the HIV-1 envelope glycoprotein complex by Rawi et al. indicated that as part of the residue pair (Gly167-Met426), Met426 might play a critical role in communicating with CD4 binding residues near Phe43 [27]. It is important to note that the docking results indicate that after positional switching from C5 to C4 of the thiazole ring, CH₂OH forms an H-bond with the backbone oxygen atom M426 (**Data not shown**), supporting that this interaction is not likely to be affected by any resistant mutation. A similar interaction was reported with DMJ-II-121, an NBD-556-type entry inhibitor [28]. Furthermore, the BMS entry inhibitors also showed developing mutations M426L in patients [29]. The Los Alamos HIV Sequence Database also show many such mutations and M426R, M426T, etc. in clinical isolates. Based on this docking result we modified NBD-14107, where we removed the CH₃ from a C4 position of thiazole, and this molecule showed excellent broad-spectrum antiviral activity [24] and switched the position of the CH₂OH group from C5 to C4 of the thiazole ring to generate **39** (NBD-14168). We synthesized and tested this molecule. The data in Table 1 demonstrate significant improvement in the antiviral potency of **39** compared to NBD-14107. Figure 2d indicated that this modification yielded an approximately 2.3-fold enhancement in antiviral potency (IC₅₀) of **39** over the parent compound NBD-14107 against HIV-1_{HXB2} in TZM-bl cells and ~4.5-fold improvement in antiviral activity against HIV-1_{III_B} in MT-2 cells. Therefore, we opted to use “positional switching of CH₂OH” as one of the major strategies in our structure-based lead optimization. We also wanted to verify whether any substitution was allowed in the CH₂OH at C4 position (**63 – 65**). It appears that a CH₃ substitution was tolerated, but etherification of CH₂OH was detrimental to the activity. We also wanted to explore the effect of the isosteric changes of thiazole S to oxazole O. This change resulted in maintaining the anti-HIV-1 activity. We would like to explore this analog further in the future.

2.2. Chemistry

The general route to NBD-compounds was described previously [16, 24]. Acids **11** and **12** were prepared as described previously [30]. Acid **4**, **7**, **10** were prepared in the analogy (Scheme 1). Amine **33** was prepared as previously described [24].

Thiazoles **15**, **17** and **21** were prepared by condensation of thioformamide with appropriate α -haloketones (Scheme 2) followed by reduction/Grignard addition and protection of hydroxyl group. In the next steps thiazoles were deprotonated using *n*-BuLi and after 1,2-

addition to previously described chiral enantiopure imines **22** or **23** [16, 24] yielded a single product (**24**, **26**, **28** or **30**, Scheme 3). TBS and *t*-BuSO groups were simultaneously cleaved using HCl solution in methanol. In the case of compound **30B** these conditions lead to undesired product **32B** (Scheme 3). However, changing the solvent to aqueous 1,4-dioxane allowed us to obtain **31** without any elimination product.

The oxazole containing compounds were synthesized using the similar sequence (Scheme 4). To prevent electrocyclic ring opening of oxazole BH₃ complex was generated before deprotonation [31].

The absolute configuration of amines **25**, **27**, **29**, **31**, **32**, **37** was not determined. In this article compounds derived from (*S*)-**22** or (*S*)-**23** were marked as **A** (e.g., **25A**) and compounds derived from (*R*)-**22** or (*R*)-**23** were marked as **B** (e.g., **25B**).

Finally, HBTU mediated amide coupling between appropriate acid (**4**, **7**, **10**, **11**, **12**) and appropriate amine (**25**, **27**, **29**, **31**, **32**, **37**, **38**) was performed followed by Pd-catalyzed deprotection (Scheme 5).

2.2.1. General—We used commercial reagents and solvents without further purification. We also performed all reactions in the air atmosphere unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) carried out on Merck TLC Silica gel plates (60 F254), using UV light for visualization and basic aqueous potassium permanganate as a developing agent. ¹H and ¹³C NMR spectra were recorded on Bruker Avance 400 instrument with operating frequency of 400 and 100 MHz, respectively. The spectra were calibrated using residual undeuterated chloroform (δH = 7.28 ppm) and CDCl₃ (δC = 77.16 ppm) or undeuterated DMSO (δH = 2.50 ppm) and DMSO-*d*₆ (δC = 39.51 ppm) as internal references. The following abbreviations are used to set multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. The purity of the final compounds was checked by LCMS in a Shimadzu LCMS-2010A using three types of detection systems such as EDAD, ELSD, and UV.

2.3. Anti-HIV-1 screening and structure-activity relationship (SAR)

In an attempt to optimize our initial lead NBD-11021, we made substantial progress in improving the antiviral activity as well as selectivity index (SI) of our next generation of inhibitors, some of which have been reported earlier [13, 15, 24, 32]. In this report, we set out to improve the antiviral activity and SI further and understand the SAR of a more advanced lead inhibitor, NBD-14107, which we recently reported [24]. We applied the hypotheses outlined above and incorporated limited substitution allowed in the aromatic ring in **Region I** (Figure 1), but we synthesized a series of molecules by removing the CH₃ group from C4 of the thiazole ring and moving the CH₂OH from C5 to C4 of the thiazole moiety. In Table 1, we compared the antiviral potency of “S” enantiomers since in most cases they yielded better antiviral activity. For example, when we evaluated the antiviral activity against HIV-1_{HXB2} in TZM-bl cells, the CH₂OH switching to C4 of thiazole in **49** (NBD-14170) enhanced the potency by ~3.4-fold and the selectivity improved by about 2.5-fold when compared with **47** (NBD-14135). Similarly, **39** showed ~2.3-fold improvement in antiviral activity and selectivity improvement was about 2.4-fold over NBD-14107. We noticed a

similar improvement when we compared **45** (NBD-14185) with **43** (NBD-14183). The presence of 4-Cl or 4-CH₃ did not make much difference. The major improvement in antiviral activity was observed with **55**, where the para position of the phenyl ring had an F and CH₂OH was moved to C4. In fact, it showed an IC₅₀ of 89 nM compared to **53** (NBD-14187) which had the antiviral IC₅₀ of 0.44 μ M, an enhancement of ~2.5-fold with an SI enhancement of ~2.4-fold. It was interesting to note that introduction of a CH₃ in the primary amine of **41** with a 4-Cl in the phenyl ring did not have much effect on antiviral activity, but apparently, the compound became more toxic. However, in a similar compound (**51** vs. **49**) with 4-CF₃ and CH₃ substitutions at the primary amine did not show any noticeable changes in both IC₅₀ and CC₅₀ values.

Interestingly, when we introduced a second CH₂OH group in position C5 of the thiazole ring (**59** – **62**), the cytotoxicity (CC₅₀) values improved substantially, but at the same time, the antiviral potency dropped considerably thereby making the SI values also poor. One notable observation was that the introduction of additional fluorine at the R₃ position did not contribute much in improving the antiviral activity or the SI. Furthermore, attaching a CH₃ in R₆ (in the primary amine) made most of the molecules more cytotoxic. We also observed that tertiary alcohol was well tolerated (**63** and **64**) but substituting CH₂OH with C(CH₃)₂OCH₃ (**65**) resulted in drastic reduction in antiviral activity. Despite the fact that most of the molecules in Table 1 are thiazole derivatives, we wanted to find out the effect of replacing thiazole with an oxazole ring on the antiviral activity and cytotoxicity. When compared **67** with **57**, we observed a ~4-fold drop in antiviral activity in both assays. Therefore, we abandoned the idea of making any CH₂OH positional switched molecules.

When we tested the NBD compounds against HIV-1 IIIB in MT-2 cells, we have seen a similar trend with the data generated against HIV-1_{HXB2} in TZM-bl cells.

2.4. The new generation of NBD-compounds inhibited HIV-1 mediated cell-cell fusion, and they showed entry antagonist traits

Two of the prerequisites of HIV-1 entry inhibitors is that they should prevent HIV-1-mediated cell-cell fusion and retain entry antagonist property. To confirm that the next generation CD4 mimics entry inhibitors possess those properties we selected three new inhibitors, **48** (NBD-14136), **39** and **55** which exhibited the best anti-HIV-1 activity and higher SI and we first evaluated their ability to inhibit HIV-1 mediated cell-cell fusion. To this end, we cocultured the indicator cells MAGI-CCR5 with the Env- and Tat-expressing HL2/3 effector cells with escalating concentrations of compounds. NBD-556 was used as a control. The cells were then cocultured with the indicator cells TZM-bl used as target cells. Our results (Figure 3A) indicated that **55** prevented HIV-1 mediated cell-cell fusion with an IC₅₀ of 9.4 $\pm$ 0.9 μ M which is similar to the IC₅₀ we detected for NBD-556 (9.1 $\pm$ 0.8 μ M). However, **39** and **48** were active at slightly higher concentration than NBD-556 and **55**.

Since we identified the HIV-1 entry inhibitor NBD-556 [11], subsequent studies reported that this small molecule could also act as entry agonist promoting CCR5 binding and enhancing HIV-1 entry into CD4-negative cells expressing CCR5 [14, 33], a particular undesirable trait. To test if our new generation inhibitors do not exhibit this undesirable trait

and behave as entry antagonist, we infected CD4-negative and CCR5-positive cells, Cf2TH-CCR5, with recombinant CD4-dependant HIV-1_{ADA} virus in the presence of different doses of the compounds. NBD-556 (a proven HIV-1 entry agonist) was used as a control. Although NBD-556 enhanced the infection of the Cf2Th-CCR5 cells, none of the new generation NBD-compounds enhanced HIV-1 infectivity in these cells as previously observed for the new generations of NBD compounds, indicating that the HIV-1 entry antagonist property is maintained in these compounds as well (Figure 3B). Additionally, the compounds did not show toxicity at the doses used in this assay.

2.5. Antiviral activity of the next generation NBD-compounds against a large panel of HIV-1 Env-pseudotyped reference viruses

Our previously described NBD compounds including NBD-14107 showed broad-spectrum antiviral activity with gradually improved IC₅₀ against a large panel of clinical isolates of different subtype [13, 16, 24]. Here we report the evaluation of the three most active new generation NBD compounds, **48**, **39** and **55** against a selection of 56 HIV-1 clones of clinical isolates of different subtype including primary and transmitted and early founder HIV-1 isolates (NIH# 11563 and 11578), and 13 recombinant HIV-1 clones. All the clinical isolates tested use CCR5 coreceptor for entry except those two that are dual tropic (CCR5/CXCR4). Transmitted viruses are known to use CCR5 for their entry into cells [34]. We compared their antiviral activity to the previously described NBD-14107. As shown in Table 2, all three compounds exhibited improved anti-HIV-1 activity compared to NBD-14107 as demonstrated by their overall means of the IC₅₀s and the respective SIs (CC₅₀/IC₅₀) determined against the panel of pseudoviruses. In fact, while the overall mean of the IC₅₀s for NBD-14107 was $0.5 \pm 0.02 \mu\text{M}$ (IC₅₀s in the range of 0.27–0.89 μM) and the SI was 79 the overall mean of the IC₅₀s determined for **48** was $0.29 \pm 0.01 \mu\text{M}$ (IC₅₀s in the range of 0.14–0.54 μM) and the calculated SI was 146.2, showing an improvement of ~1.7 and 1.8 folds, respectively. **39** showed a 2.8-fold improvement in the overall mean of the IC₅₀s relative to NBD-14107 ($0.18 \pm 0.008 \mu\text{M}$ with the IC₅₀s in the range of 0.094–0.35 μM) and a ~3 fold improvement of the SI. Finally, the overall mean of the IC₅₀s determined for **55** was of $0.11 \pm 0.004 \mu\text{M}$ (IC₅₀s in the range of 0.063–0.18 μM) indicating a 4.5-fold improvement when compared with NBD-14107, but the SI for this compound showed a 2.5 fold improvement. Based on the SI values calculated for each subtype, we observed that NBD-14107 had better activity against the A_{recombinant} pseudoviruses while **48** had better activity against the subtype C. **39** was equally active against the A_{recombinant} pseudoviruses and the subtype C. Finally, **55** showed the best activity against subtypes A, C, and D-D/A. Furthermore, these three new compounds were active against all the clinical isolates regardless of the viral subtype similar to what we previously reported for NBD-14107 [24] indicating that these compounds have broad-spectrum inhibitory activity against HIV-1. Moreover, **48**, **39**, and **55** were poorly active against the control pseudovirus VSV-G tested in U87-CD4-CXCR4 cells suggesting that the inhibitory activity of these compounds is specific to HIV-1. Additionally, these compounds did not induce any toxicity in this cell line.

2.6. NBD compounds inhibited cell-to-cell HIV-1 transmission

The HIV-1 transmission from an infected donor T cell to an uninfected target T cell occurs through a cell-cell adhesion formation known as virological synapse (VS). In vitro studies

indicate that the HIV-1 cell-to-cell transmission is far more efficient than the HIV-1 cell-free infection [35, 36]. In fact, the VS may facilitate the transfer of multiple viral particles at the same time into the non-infected cell. It has been reported that cell-to-cell HIV-1 transmission in vitro is resistant to potent broadly neutralizing antibodies including CD4 binding site (CD4bs) antibodies [35, 37, 38], but not to antiretroviral agents including some entry inhibitors [39]. Based on these findings, we evaluated the NBD compounds against the cell-to-cell HIV-1 transmission using two different systems. In the first system, we used chronically infected cells: H9 cells infected with HIV-1_{IIIB} (CXCR4-tropic) and MOLT-4 cells infected with HIV-1_{ADA} (CCR5-tropic). BMS-626529 was used as a control. As reported in Table 3A, the inhibitory activity of all the compounds including BMS-626529 was higher against HIV-1_{IIIB} than against HIV-1_{ADA}. In fact, the IC₅₀s against the HIV-1_{IIIB} in this assay were comparable to those determined in the cell-free infection assay reported above. In the second system, we used PBMC cells acutely infected with three laboratory-adapted HIV-1 clones and ten primary HIV-1 clinical isolates of different subtype and coreceptor usage. We also evaluated the toxicity of these compounds in uninfected PBMCs (Table 3B). All the NBD compounds inhibited the cell-to-cell HIV-1 transmission against all the HIV clones except one of the primary clinical isolates, HIV-1_{CMU02} that displayed some resistance as suggested by the higher IC₅₀ (Table 3B). We calculated the overall mean of the IC₅₀s obtained for the NBD compounds against the 13 viruses and found that was about 3-fold higher than the overall means obtained for the NBD compounds against the panel of HIV-1 Env-pseudotyped reference viruses used in the cell-free infection assay. Moreover, the viral clone HIV-1_{CMU02} and the two subtype O clones HIV-1_{BCF01} and HIV-1_{BCF03} were resistant to the potent entry inhibitor BMS-626529. Our findings were consistent with previous reports which showed that HIV-1 isolates subtypes AE and O were not susceptible to the BMS compounds [40].

2.7. Drug sensitivity to Env-mutated HIV-1_{HXB-2} pseudoviruses

To evaluate the binding mode of the NBD compounds, we prepared a series of mutant pseudoviruses HIV-1_{HXB-2} carrying amino acid substitutions in the Env gp120 region (Table 4). Some of the amino acid substitutions were described previously. For example, M426L, M434I, M434I/I595F and M475I were reported in the resistance study performed with BMS-378806 [41]. The substitutions L116P and A204D were described in the resistance study performed with BMS-626529 [29]. The S375H substitution is common to subtype CRF01_AE and subtype O HIV-1 viruses and conferred resistance to BMS-378806 and BMS-626529 [1, 29].

The NBD compounds were tested against the wild-type (WT) HIV-1_{HXB2}, used as a reference and the mutant pseudoviruses to verify if these amino acid substitutions would generate HIV-1 resistance to the compounds. Consistent with previous studies, we observed that the L116P and the A204D substitutions conferred high resistance to BMS-626529 [29] (Table 4). The L116P substitution did not affect the antiviral activity of the three NBD compounds as indicated by the low IC₅₀ detected that was similar to the IC₅₀ obtained against the WT HIV-1_{HXB2}. However, the A204D substitution located in the V2 induced a 6-fold and 5-fold increase in the IC₅₀ of **39** and **55**, respectively, but this substitution had no impact on the **48** IC₅₀. A second amino acid substitution which affected the activity of **39**

and **55** was the I424F located in the CD4 binding site. In fact, we detected a 9-fold and 7-fold increase in their IC₅₀s respectively, when compared to the IC₅₀s obtained against the WT HIV-1_{HXB2}. The I424F substitution also induced resistance to BMS-626529. All the variation of the S375 conferred resistance to BMS-626529 but not to the NBD compounds. Moreover, the only substitution inducing HIV-1 resistance to **48** with a 4-fold increase in the IC₅₀ was the N425K also located in the CD4-binding site. One important observation from the sensitivity study also confirmed that the M426L mutation did not affect the sensitivity of NBD compounds tested. That corroborates what we inferred from the docking study that M426 mutation might not have any effect on drug resistance since the CH₂OH forms an H-bond with the backbone O atom of M426. Finally, the remaining substitutions analyzed in this study did not induce HIV-1 resistance to the NBD-compounds. Taken together these results suggest that the NBD compounds may interfere with the gp120/CD4 interaction preventing HIV-1 infection.

The data in Table 4 also demonstrate that the binding sites of NBD compounds and BMS compounds are different which was confirmed by X-ray structure of BMS compounds with the HIV-1 gp120 trimmer by Pancera et al. [1].

2.8. In Vitro ADMET

In vitro ADMET and *in vivo* PK studies of drug molecules during the initial discovery phase is critical to reducing the attrition rate in late-stage drug development [42, 43]. In 1997, approximately 39% of the drug failures were allegedly due to poor ADMET [44]. However, that alarming trend dropped significantly [42] after pharmaceutical companies started conducting ADMET and PK studies in the early drug development stage as part of the “Fail early, fail cheap” strategy [42]. The discovery and development of a new drug are time-consuming (12–15 years) and expensive (more than a billion dollars). Therefore, we have also adopted this strategy in our drug discovery efforts and evaluated the *In vitro* ADMET properties of **39** and **55**, the two most active compounds and compared the data with those of BMS-626529. The knowledge gained from these studies will provide critical information for further optimization of the inhibitors.

The *in vitro* ADMET results presented in Table 5 show that **39** and **55** have slightly better aqueous solubility profiles (100 to 500 µM) than BMS-626529 (<100 µM) at a DMSO concentration of 2%, which improved with an increase in DMSO concentration to 5%. In the Caco-2 bidirectional permeability assay, which predicts the human intestinal permeability of orally administered compounds, **39** and **55** were similarly permeable but had lower apparent permeability constants compared with BMS-626529. All three compounds had efflux ratios that were greater than 2, indicating the potential involvement of an efflux transporter that could transport compound from the basolateral side, back to the apical side. The efflux ratios for all three compounds were also reduced in the presence of the P-glycoprotein (P-gp) inhibitor verapamil. When the efflux ratio is reduced to less than 2 in the presence of a P-gp inhibitor, the compound is likely to be a substrate of P-gp. In the case of BMS-626529, the efflux ratio was reduced from 3.46 to 1.69 in the presence of the P-gp inhibitor verapamil, suggesting the possible involvement of P-gp. For **39** and **55**, the efflux ratios were significantly reduced in the presence of verapamil (from 36.4 to 13.3 for **39**; from 30.5 to

14.4 for **55**). However, they were not reduced to less than 2, suggesting that other efflux transporters, such as BCRP, MRP2, etc., may be involved [45].

We studied the metabolic stability of our molecules in human liver microsomes because the liver is the primary drug metabolism organ. **39** and **55** were both stable when incubated with human liver microsomes for 120 min (88.5 to 90.4% of compound remaining), while BMS-629529 showed some metabolism (71.5% of compound remaining). Furthermore, based on the *in vitro* human intrinsic clearance data (CL_{int}), we can classify all NBD molecules and BMS-626529 as low-clearance compounds. The NBD compounds were also tested for their human plasma protein binding potential and compared with BMS-626529. **39** and **55** were both highly bound to proteins in human plasma (>99%), while BMS-629529 was 86.9% bound (Table 5).

The three compounds were also evaluated for their ability to inhibit the activity of seven human CYP450 isoforms (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) that metabolize more than 80% of all drugs, to predict the potential for drug-drug interactions with other co-administered therapeutics. When tested up to 25 μ M, none of the compounds inhibited any of the CYP isoforms.

We also evaluated the potential of these molecules to inhibit the hERG potassium ion channel, a known indicator of the development of acquired long QT syndrome that might lead to fatal ventricular arrhythmia, such as Torsade de Pointes. The effect of the three compounds on cloned hERG potassium channel expressed in mammalian cells was studied. BMS-626529 and **55** showed weak or no inhibition of the hERG current, with an IC_{50} of 21.9 μ M and 3.0 μ M, respectively. **39** inhibited the hERG channel with an IC_{50} of 0.9 μ M which might be pharmacologically relevant (Table 5). Cisapride (90 nM), the positive control, inhibited hERG current (Mean $\pm$ SD; n = 2) by $85.2 \pm 2.3\%$, which was consistent with historical data. It is pertinent here to mention that many drugs were withdrawn from the market due to cardiac toxicities, but all had nM level hERG inhibitory activity. Nevertheless, these data suggest that there is room for improvement of these NBD series inhibitors.

3. Conclusion

We presented our continued effort to optimize our lead entry antagonist reported earlier by structure-based drug design approach. We have synthesized thirty novel molecules to validate our working hypothesis, experimentally evaluated their antiviral activity, and determined their mechanism of action and measured the *in vitro* ADMET profiles. We conclusively showed that switching CH_2OH from C5 to a C4 position in the thiazole ring resulted in noticeable enhancement of antiviral activity and SI. One of the optimized molecules, **55**, had a remarkable IC_{50} of 89 nM against the HIV-1_{HXB2} pseudovirus. We tested **39** and **55** against a large panel of 55 HIV-1 Env-pseudotyped viruses representing clinical isolates of diverse subtypes, and the data showed these molecules have the broad-spectrum antiviral activity. The drug sensitivity study confirmed that these molecules bind to the CD4 binding site in gp120. The *in vitro* ADMET data also suggest that these NBD series molecules have similar profiles as that of the control attachment inhibitor BMS-626529. However, there is some room for improvement to make them more clinically relevant drug

candidates. The presented data is expected to broaden our knowledge and help to optimize these molecules further to clinically relevant candidate drugs.

4. Experimental Section

4.1. Cells and viruses

MT-2 cells [46], TZM-bl cells [47], U87CD4+CXCR4+ cells [48] and HL2/3 cells [49] were obtained through the NIH ARP. HEK 293T cells were purchased from ATCC. CD4-negative Cf2Th-CCR5+ cells and Env expression vector pSVIIIenv-ADA were kindly provided by Dr. J. G. Sodroski [50]. The human PBMC (Peripheral blood mononuclear cell) were isolated from buffy coats of healthy HIV-1 negative donor obtained from the New York Blood Center (New York, NY) and grown in RPMI 1640 medium supplemented with fetal bovine serum (FBS) penicillin and streptomycin. The PBMC were stimulated with 5 µg/mL phytohemagglutinin (PHA) and 20 U/mL interleukin 2 (IL-2) (Sigma).

HIV-1 Env molecular clone expression vector pHXB2-env (X4) DNA was also obtained through the NIH ARP [51]. HIV-1 Env molecular clones of gp160 genes for HIV-1 Env pseudovirus production were obtained as follows: clones representing the standard panels A, A/D, A2/D, D and C (QB099.391M.Env.B1) were obtained through the NIH ARP from Dr. J. Overbaugh [52, 53]. The HIV-1 Env molecular clones panel of subtype A/G and A/E (CRF01_AE clone 269) Env clones were obtained through the NIH ARP from Drs. D. Ellenberger, B. Li, M. Callahan and S. Butera [54]. The AE clone AA058 was kindly provided by Drs. R. J. McLinden and A. L. Chenine from US Military HIV Program, Henry M. Jackson Foundation (Silver Spring, MD). The HIV-1 Env panel of standard reference subtype B Env clones was obtained through the NIH ARP from Drs. D. Montefiori, F. Gao and M. Li (PVO, clone 4 (SVPB11); TRO, Clone 11 (SVPB12); AC10.0, clone 29 (SVPB13); QH0692, clone 42 (SVPB6); SC422661, clone B (SVPB8)); from Drs. B. H. Hahn and J. F. Salazar-Gonzalez (pREJO4541, clone 67 (SVPB16); pRHPA4259, clone 7 (SVPB14)); from Drs. B. H. Hahn and D. L. Kothe (pTHRO4156 clone 18 (SVPB15), pCAAN5342 clone A2 (SVPB19)) [55, 56]. The subtype B clones pWEAUd15.410.5017, p1058_11.B11.1550, p1054.TC4.1499, p9014_01.TB1.4769 and p9021_14.B2.4571 were obtained through the NIH ARP from Drs. B. H. Hahn, B. F. Keele and G. M. Shaw [57]. The subtype C HIV-1 reference panel of Env clones were also obtained through the NIH ARP from Drs. D. Montefiori, F. Gao, S. A. Karim and G. Ramjee (Du 156.12; Du172.17); from Drs. D. Montefiori, F. Gao, C. Williamson and S. A. Karim (Du422.1), from Drs. B. H. Hahn, Y. Li and J. F. Salazar-Gonzalez (ZM197M.PB7; ZM214M.PL15, ZM249M.PL1); from Drs. E. Hunter and C. Derdeyn (ZM53M.PB12; ZM109F.PB4); from Drs. L. Morris, K. Mlisana and D. Montefiori, (CAP210.2.00.E8) [58–60]. The HIV-1 Subtype C Panel of Indian gp160 Env Clones HIV-16055-2 clone 3, HIV-16936-2 clone 21, HIV-25711-2 clone 4, HIV-225925-2 clone 22 and HIV-26191-2, clone 48 were obtained through the NIH ARP from Drs. R. Paranjape, S. Kulkarni and D. Montefiori [54]. HIV-1 Env molecular clones MF535.W0M.Env.C1 and BF535.W6M.Env.A1 of subtype D/A were obtained through the NIH ARP from Dr. J. Overbaugh [61]. The Env pseudotyped genes of BG505.T332N, KNH1144, and B41 were kindly provided by Dr. J. P. Moore of the Weil Cornell Medical College, NY.

The Env-deleted proviral backbone plasmids pNL4-3.Luc.R-E-DNA (from Dr. N. Landau) [62, 63] and the pSG3^{env} DNA (from Drs. J. C. Kappes and X. Wu) [47, 56] were obtained through the NIH ARP. MLV gag-pol-expressing vector pVPack-GP, Env-expressing vector pVPack-VSV-G and a pFB-Luc vector were obtained from Stratagene (La Jolla, CA). HIV-1_{IIIB} laboratory-adapted strain was obtained through the NIH ARP.

4.2. Pseudovirus preparation

Pseudoviruses capable of single cycle infection were prepared as previously described [14, 16]. Briefly, 5×10^6 HEK293T cells were transfected in a 15 ml volume of a solution containing 10 µg of an HIV-1 Env-deleted pro-viral backbone plasmid pSG3^{env} or pNL4-3.Luc.R-E-DNA, and 10 µg of an HIV-1 Env-expression plasmid with FuGENE 6 (Roche). VSV-G pseudovirus was prepared by transfecting the HEK293T cells with a combination of the Env-expressing plasmid pVPack-VSV-G, the MLV gag-pol-expressing plasmid pVPack-GP, the pFB-Luc plasmid and FuGENE 6. Pseudovirus-containing supernatants were collected two days after transfection, filtered, tittered and stored in aliquots at -80°C .

4.3. Measurement of antiviral activity

4.3.1. Single-cycle infection assay in TZM-bl cells—The antiviral activity of the NBD compounds was evaluated in single-cycle infection assay by infecting TZM-bl cells with an HIV-1 pseudovirus expressing the Env from the lab-adapted HIV-1_{HXB-2} (X4). Additionally, NBD-14107, **48**, **39**, and **55** were also tested against a large group of HIV-1 pseudotyped viruses expressing the Env from the panel of clinical isolates as previously described [14, 16]. Briefly, TZM-bl cells were plated at 1×10^4 / well in a 96-well tissue culture plate and cultured at 37°C overnight. On the following day, aliquots of HIV-1 pseudovirus were pre-treated with graded concentrations of the small molecules for 30 min, added to the cells and incubated for 3 days. Cells were washed and lysed with 50 µl of lysis buffer (Promega). 20 µl of the lysates were transferred to a white plate and mixed with the luciferase assay reagent (Promega). The luciferase activity was measured immediately with a Tecan infinite M1000 reader, and the percent inhibition by the compounds and IC₅₀ (the half maximal inhibitory concentration) values were calculated using the GraphPad Prism software.

4.3.2. Single-cycle infection assay in U87-CD4-CXCR4 cells—NBD-14107, **48**, **39**, and **55** were tested against control pseudovirus VSV-G, obtained as described above and against HIV-1_{HXB-2}, wild-type (WT) pseudovirus or the HIV-1_{HXB-2} Env-mutated pseudoviruses carrying a single or double amino acid substitution. Briefly, U87-CD4-CXCR4 cells were plated in a 96-well tissue culture plate at 1×10^4 / well and cultured overnight. The following day, aliquots of VSV-G pseudovirus or mutated pseudovirus pre-treated with graded concentrations of the small molecules for 30 min were added to the cells and incubated for 3 days. Cells were washed and lysed with 40 µl of lysis buffer. The lysates were then transferred to a white plate and mixed with the luciferase assay reagent. The luciferase activity was immediately measured to calculate the percent of inhibition and IC₅₀ values by using the GraphPad Prism software.

4.3.3. Multi-cycle infection assay in MT-2 cells—The NBD small molecules were evaluated against the full-length laboratory-adapted HIV-1_{IIIB} as previously described [64]. Briefly, aliquots of HIV-1_{IIIB} at 100 TCID₅₀ were pre-incubated with an equal volume of graded concentrations of compounds for 30 min then added to the MT-2 cells at 1×10^4 /well in a 96-well tissue culture plate. The following day, the culture supernatants were replaced with fresh media. Four days post-infection, the supernatants were collected and mixed with an equal volume of 5 % Triton X-100 and tested for p24 antigen by sandwich-ELISA. The GraphPad Prism software was used to calculate the percent inhibition of p24 production and IC₅₀ values.

4.4. Evaluation of cytotoxicity

4.4.1. TZM-bl cells and U87-CD4-CXCR4 cells—The cytotoxicity of the small molecules in TZM-bl and U87-CD4-CXCR4 cells was measured by the colorimetric XTT method as previously described [13]. Briefly, the cells were plated in a 96-well tissue culture plate at 1×10^4 / well and cultured at 37 °C. Following overnight incubation, the cells were incubated with 100 µl of the compounds at graded concentrations and cultured for 3 days. The XTT solution was added to the cells, and 4 h later the soluble intracellular formazan was quantitated at 450 nm. The percent of cytotoxicity and the CC₅₀ (the concentration for 50 % cytotoxicity) values were calculated as above.

4.4.2. MT-2 cells—The cytotoxicity of the NBD small molecules was also measured in MT-2 cells with the colorimetric XTT method as previously described [64]. Briefly, 100 µl of a small molecule at graded concentrations was added to an equal volume of cells (10^5 cells/ml) in 96-well plates. The following day, the culture supernatants were replaced with fresh media and incubated for 4 days. Four hours after the addition of XTT the soluble intracellular formazan was quantitated, and the percent of cytotoxicity and the CC₅₀ values were calculated as above.

4.4.3. Cf2Th-CCR5 cells—The cytotoxicity of the small molecules in Cf2Th-CCR5 cells was also measured by the colorimetric XTT method. Briefly, Cf2Th-CCR5 cells were plated in a 96-well plate and cultured at 37 °C overnight. Next, the cells were incubated with 100 µl of the compounds at graded concentrations and cultured for 48 h. The XTT solution was added to the cells, and 4 h later the soluble intracellular formazan was quantitated to calculate the percent of cytotoxicity and the CC₅₀ values as above.

4.4.4. Uninfected PBMC cells—The cytotoxicity of the small molecules in freshly isolated and stimulated PBMC cells was measured by the colorimetric XTT method. 50 µl of uninfected PBMC cells were pre-treated with the same volume of escalating concentrations of compounds for 1 h at 37 °C before adding 100 µl of the complete medium. The cells were then cultured for 48 h. The XTT solution was added to the cells, and 4 h later the soluble intracellular formazan was quantitated to calculate the percent of cytotoxicity and the CC₅₀ values

4.5. HIV-1 mediated cell-cell fusion inhibition assay

The HIV-1 mediated cell-cell fusion assay was performed as previously described [13, 65, 66] with some modifications. The indicator cells TZM-bl, a HeLa cell line which expresses luciferase under control of HIV-1 Tat were used as target cells and the HL2/3 cells, a HeLa cell line which does not produce mature virions but expresses HIV-1_{HXB2} Env on the surface and Tat, Gag, Rev and Nef proteins in the cytoplasm as effector cells. Briefly, the HL2/3 cells were plated in a 96-well plate at 1×10^4 / well and incubated with escalating concentrations of NBD-compounds for 1 h. The TZM-bl cells were then added to the culture at 1×10^4 cells/well and cultured for 24 h. The cells were washed with PBS and lysed. The luciferase activity was immediately measured as reported above to calculate the percentage of inhibition of the luciferase expression and the IC₅₀ values.

4.6. Assay in Cf2Th-CCR5 cells

CD4-negative Cf2Th-CCR5 cells were plated at 6×10^3 cells/well in a 96-well tissue culture plate and incubated overnight. The cells were infected with the luciferase-expressing recombinant CD4-dependent pseudovirus HIV-1_{ADA} as previously described [12]. Briefly, following overnight incubation, aliquots of HIV-1_{ADA} pseudovirus pre-treated with graded concentrations of the small molecules for 30 min were added to the cells and cultured for 48 h. Cells were washed with PBS and lysed with 40 μ l of cell lysis reagent. Lysates were transferred to a white 96-well plate and mixed with 100 μ l of luciferase assay reagent. The luciferase activity was immediately measured to obtain the relative infection compared to the untreated control. The Relative virus infectivity indicates the amount of infection detected in the presence of the compounds divided by the amount of infection detected in the absence of the compounds.

4.7. Cell-to-Cell HIV-1 Transmission

4.7.1 Chronically infected cells /TZM-bl system—The cell-to-cell HIV-1 transmission inhibition assay was performed as previously described [65, 67] with some modifications. Briefly, target TZM-bl cells were plated at 10^4 /well in a 96 well plate 24 h before the experiment. As transmitting cells, for the CXCR4-tropic assay we used chronically infected H9/HIV-1_{IIIB} at 2×10^3 cells/well, and for the CCR5-tropic assay, we used MOLT-4/CCR5 cells chronically infected with HIV-1_{ADA} at 4×10^3 cells/well. The cells were treated with 200 μ g/mL mitomycin C (Sigma) for 1 h at 37 °C, washed with PBS, and incubated with the target cells and escalating concentrations of drugs for 48 h. Then, the cells were washed and lysed. The lysates were transferred to a white plate and mixed with the luciferase assay reagent. The luciferase activity was immediately measured to calculate the percent of inhibition and IC₅₀ values by using the GraphPad Prism software.

4.7.2. Acutely infected PBMC / TZM-bl system—The cell-to-cell HIV-1 transmission inhibition assay was performed as previously described [35] with some modifications. Briefly, target TZM-bl cells were plated at 10^4 /well in a 96 well plate 24 h before the experiment. PBMC were infected with the replication-competent virus in the presence of 30 μ g/ml of DEAE-dextran hydrochloride (Sigma). On the 4th-day post-infection the cells were washed to remove the free virus and counted. 2×10^4 /well infected PBMC cells were pre-

treated with escalating concentrations of compounds for 1 h at 37 °C before co-culturing with the TZM-bl cells. PBMC and TZM-bl were co-cultured for 48 h. Then,, the TZM-bl cells were washed and lysed. The lysates were then transferred to a white plate to immediately measure the luciferase activity to calculate the percent of inhibition and IC₅₀ values by using the GraphPad Prism software.

4.8. Drug sensitivity of ENV-mutated pseudovirus

The amino acid substitutions were introduced into the pHXB2-Env expression vector by site-directed mutagenesis (Stratagene) using mutagenic oligonucleotides. The viral DNA sequences were confirmed by sequencing the entire ENV gene of each construct and analyzed with the Geneious R8 software (Biomatters, New Zealand). We infected U87-CD4-CXCR4 cells with the ENV-mutated pseudoviruses pretreated for 30 min with varying concentrations of the NBD compounds and incubated for 3 days to measure the activity of the compounds against pseudoviruses expressing different amino acid substitutions. Cells were washed with PBS and lysed with 40 µl of cell culture lysis reagent. Lysates were transferred to a white 96-well plate and mixed with 100 µl of luciferase assay reagent. We immediately measured the luciferase activity to calculate the IC₅₀, as described above.

4.9. ADMET Study

4.9.1. Aqueous Solubility—Solubility was determined using the Millipore MultiScreen® solubility filter plate method (Burlington, MA). 39, 55, and BMS-626529 dissolved in DMSO were diluted to 500 µM in universal aqueous buffer (pH 7.4, prepared according to the Millipore Protocol Note PC2445EN00) with 2 or 5% DMSO final, and incubated in a MultiScreen® solubility filter plate in quadruplicate for 90 min with shaking at room temperature. Following incubation, the samples were vacuum filtered, an aliquot of each filtrate was transferred to a 96-well UV plate and mixed with acetonitrile (20% acetonitrile final). The absorbance of the test compounds was measured at six wavelengths using a Molecular Devices SPECTRAmax® 190 microplate spectrophotometer and Softmax® Pro 5.4 software (Sunnyvale, CA). The relative solubility in the form of a ratio of filtrate versus standard was calculated. Based on the calculated ratios, the solubility was predicted to be approaching the upper limit of 500 µM, between 100 and 500 µM, or likely to be less than 100 µM.

4.9.2. Metabolic Stability—Human liver microsomes were purchased from Corning Life Sciences (Woburn, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). 39, 55, and BMS-626529 (3 µM final) were incubated in duplicate with pooled mixed gender human liver microsomes (0.5 mg/ml) and cofactors (2.5 mM NADPH and 3.3 mM magnesium chloride) in 0.1M phosphate buffer, pH 7.4, at 37°C. Aliquots were removed at several time points (0, 15, 30, 60, 90 and 120 min), and mixed with acetonitrile containing internal standard. Samples were analyzed by LC-MS/MS, and the percentage of parent test compound remaining at each time point was determined by comparison with the zero minute time point. Using these results, *in vitro* half-life ($t_{1/2}$, min) was calculated using the equation: $t_{1/2} = -0.693/k$, where k was the slope of the linear regression from the natural log of the % remaining data versus time. Intrinsic clearance (CL_{int}, µl/min/mg) was calculated with the formula: $CL_{int} = (0.693/t_{1/2}) / \text{protein concentration}$.

4.9.3. CYP Inhibition—All substrates, inhibitors, and cofactors were purchased from Sigma-Aldrich (St. Louis, MO). The inhibitory effect of the test compounds (0.1, 1, 2.5, 10, and 25 μM) was tested on various CYP isoforms in human liver microsomes (Corning Life Sciences). **39**, **55**, and BMS-626529 were incubated for 20 min at 37°C with microsomes (0.5 mg/ml), cofactors (2.5 mM NADPH and 3.3 mM MgCl_2), and a substrate cocktail consisting of 25 μM phenacetin (CYP1A2), 25 μM bupropion (CYP2B6), 5 μM paclitaxel (CYP2C8), 10 μM diclofenac (CYP2C9), 20 μM mephenytoin (CYP2C19), 10 μM bufuralol (CYP2D6), 50 μM testosterone (CYP3A4), and 4 μM midazolam (CYP3A4), in 100 mM phosphate buffer, pH 7.4 (0.3% DMSO final). Positive control incubations containing known inhibitors of the CYPs of interest were also included. Samples were analyzed by LC-MS/MS for the formation of metabolites of the specific probe substrates. The formation of metabolites in the presence of test compounds was compared to metabolites formed in the absence of test compounds, and an IC_{50} (concentration of compound that produces 50% inhibition) was calculated if sufficient inhibition was observed.

4.9.4. Caco-2 Bidirectional Permeability with P-gp Determination—Caco-2 cells grown in tissue culture flasks were trypsinized, suspended in media, and a known concentration of cell suspensions was seeded onto wells of a Millipore 96-well Caco-2 plate. The cells were allowed to grow and differentiate for three weeks, replacing media at 2-day intervals. Test compounds were prepared in transport buffer, pH 7.4 (1.98 g/L glucose in 10 mM HEPES, 1 $\times$ Hank's Balanced Salt Solution) in the presence or absence of 100 μM verapamil as a P-gp chemical inhibitor. For Apical to Basolateral (A–B) permeability, solutions of **39**, **55**, and BMS-626529 were added to the apical side, while the test compound solutions were added to the basal side for B–A transport. Following incubation for 2 h, the donor and receiver side buffers were removed for analysis by LC-MS/MS. To verify tight junctions and monolayer integrity, the A-side buffer contained 100 μM lucifer yellow dye in Transport Buffer at pH 7.4, while the B-side buffer was Transport Buffer, pH 7.4, and aliquots of the cell buffers were analyzed by fluorescence (Lucifer yellow transport 2%).

The apparent permeability coefficient (P_{app} , $\times 10^{-6}$ cm/sec) was calculated using the following equation: $P_{\text{app}} = (dQ/dt) / (C_0 \times A)$, where dQ/dt was the rate of permeation, C_0 was the initial concentration of the test compounds, and A was the area of the cell monolayer. Using the mean P_{app} values, efflux ratios (ER) were calculated using the following equation: $\text{ER} = P_{\text{app(B} \rightarrow \text{A)}} / P_{\text{app(A} \rightarrow \text{B)}}$. An ER greater than 2 indicates that a compound is a potential substrate for P-gp or other active efflux transporter(s). A significant decrease in the efflux ratio in the presence of verapamil suggests the test compound is a substrate for P-gp.

4.9.5. Plasma Protein Binding—Binding of **39**, **55**, and BMS-626529 (5 μM final) to proteins in human plasma was performed by equilibrium dialysis using the Pierce Rapid Equilibrium Dialysis (RED) device (Waltham, MA). Test compounds were added in duplicate to plasma, and then dialyzed in a RED device against PBS, as per the manufacturers' instructions, and incubated on an orbital shaker. At the end of the incubation, aliquots from both plasma and PBS sides were collected, and an equal amount of PBS was

added to the plasma sample, and an equal volume of plasma was added to the PBS sample. Methanol containing an analytical internal standard (IS) was added to precipitate the proteins and release the test article. After centrifugation, the supernatant was transferred to a new plate and analyzed by LC-MS/MS to obtain peak area ratios (analyte/IS) for determining the fraction unbound. The extent of binding was reported as a fraction unbound (f_u) value, which was calculated using the equation: $f_u = PF/PC$, where PC is the test compound in the protein-containing compartment, and PF is the test compound in the protein-free compartment.

4.9.6. hERG Channel Inhibition (IC₅₀ Determination)—The *in vitro* effects of **39**, **55**, and BMS-626529 on the hERG (human ether-à-go-go-related gene) potassium channel current (a surrogate for I_{Kr} , the rapidly activating delayed rectifier cardiac potassium current) was examined. The cells used were HEK293 (human embryonic kidney) cells stably transfected with hERG cDNA. Stable transfectants were selected by coexpression with the G418-resistance gene incorporated into the expression plasmid. Cells cultured in a solution (Dulbecco's Modified Eagle Medium with fetal bovine serum, penicillin G sodium, streptomycin sulfate and G418) were transferred to the recording chamber and superfused with vehicle control solution (HB-PS + 0.3% DMSO). The recording was performed using a combination of in-line solution pre-heater, chamber heater, and feedback temperature controller. A commercial patch clamp amplifier was used for whole-cell recordings. Cells stably expressing hERG were held at -80 mV. Onset and steady-state inhibition of hERG potassium current due to the test compounds was measured using a pulse pattern with fixed amplitudes (conditioning prepulse: $+20$ mV for 1 s; repolarizing test ramp to -80 mV (-0.5 V/s) repeated at 5 s intervals). Each recording ended with a final application of a supramaximal concentration of the reference substance (E-4031, 500 nM), to assess the contribution of endogenous currents. The remaining uninhibited current was subtracted off-line digitally from the data to determine the potency of the test substance for hERG inhibition.

The concentration-response relationship was evaluated at a near-physiological temperature (33 to 35°C). Test article concentrations were prepared fresh daily by diluting the appropriate stock solutions into HB-PS (with a final concentration of DMSO at 0.3% v/v). For each test article, four concentrations were selected to evaluate the concentration-response relationship, and each concentration was tested in at least three cells ($n = 3$). The positive control, cisapride, was tested in two cells ($n=2$). One or more test compound concentrations were applied sequentially (without washout between test substance concentrations) in ascending order, to each cell ($n = 3$). Peak current was measured during the test ramp. A steady state was maintained for at least 30 s before applying test article or positive control. Peak current was measured until a new steady state was achieved.

The steady state before and after test article application was used to calculate the percentage of current inhibited at each concentration. Concentration-response data were fit to an equation of the form:

$$\% \text{ Inhibition} = \{1 - 1/[1 + ([\text{Test}]/\text{IC}_{50})^N]\} * 100,$$

where [Test] was the test article concentration, IC_{50} was the test article concentration at half-maximal inhibition, N was the Hill coefficient and % Inhibition was the percentage of current inhibited at each test article concentration.

5.0. Chemistry

5-Bromo-1,3-difluoro-2-methylbenzene (1)

To a solution of diisopropylamine (33 mL, 235 mmol, 1.4 equiv) in THF (166 mL) *n*-BuLi in hexane (2.5 M, 86 mL, 215 mmol, 1.3 equiv) was added dropwise at -20°C under a constant flow of argon. The resulting mixture was cooled to -90°C and at this temperature a solution of 1-bromo-3,5-difluorobenzene (19 mL, 165 mmol) in THF (166 mL) was added dropwise. The reaction mixture was stirred for 15 minutes, and a solution of methyl iodide (21 mL, 337 mmol, 2 equiv) in THF (38 mL) was added dropwise. The temperature of the resulting mixture was elevated to room temperature, and water was added (~ 0.5 L). The resulted solution was extracted with CH_2Cl_2 (3×100 mL). The combined organic layers were dried over Na_2SO_4 , filtered and evaporated. The residue was distilled at reduced pressure (bp = 120°C , ~ 200 torr). $M = 24.0$ g. Yield = 70%.

^1H NMR: (CDCl_3 , 400 MHz) δ = 2.14 (t, $J = 1.6$ Hz, 3 H), 7.03 (d, $J = 6.4$ Hz, 2 H)

^{13}C NMR: (CDCl_3 , 100 MHz) δ = 7.0 (t, $J = 3.7$ Hz), 113.0 (t, $J = 21.2$ Hz), 114.9 (dd, $J = 20.5$, 8.8 Hz, 2C), 118.6 (t, $J = 12.4$ Hz), 161.6 (dd, $J = 249.6$, 10.3 Hz, 2C).

2-(3,5-Difluoro-4-methylphenyl)-1H-pyrrole (2)

1 (24.0 g, 116 mmol), pyrrole (24.0 mL, 343 mmol, 3 equiv), KOAc (34.0 g, 346 mmol, 3 equiv), and $[\text{PdCl}(\text{C}_3\text{H}_5)]_2$ (~ 1 g) were dissolved in DMA (347 mL) under a constant flow of N_2 . The reaction mixture was stirred at reflux for 8 h, then cooled to room temperature, poured into H_2O (1 L), and extracted with Et_2O (5×100 mL). The combined organic phases were evaporated in vacuo. The residue was extracted with DCM. The organic phase was dried over Na_2SO_4 , filtered and evaporated. The residue was used without further purification. $M = 16.76$ g. Yield = 75%.

^1H NMR: (CDCl_3 , 400 MHz) δ = 2.18 (t, $J = 1.8$ Hz, 3 H), 6.29 (dd, $J = 6.0$, 2.6 Hz, 1 H), 6.49 – 6.53 (m, 1 H), 6.85 – 6.88 (m, 1 H), 6.98 (d, $J = 8.6$ Hz, 2 H), 8.99 (br. s, 1 H).

1-(5-(3,5-Difluoro-4-methylphenyl)-1H-pyrrol-2-yl)-2,2,2-trifluoroethanone (3)

Crude pyrrole **2** (16.76 g, 86.8 mmol) was dissolved in CH_2Cl_2 (86 mL) and pyridine (8.42 mL, 104.1 mmol, 1.2 equiv) was added followed by dropwise addition of TFAA (14.5 mL, 104 mmol, 1.2 equiv). After completion of the addition, the mixture was stirred for 1 h and the precipitate was filtered and washed with CH_2Cl_2 . $M = 7.19$ g. Yield = 29%.

^1H NMR: (DMSO , 400 MHz) δ = 2.14 (s, 3 H), 7.02 (d, $J = 4.3$ Hz, 1 H), 7.25 (ddd, $J = 4.2$, 4.0, 2.1 Hz, 1 H), 7.75 (d, $J = 8.7$ Hz, 2 H), 12.92 (br. s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 6.7 (t, J = 3.2 Hz), 108.7 (dd, J = 20.1, 8.8 Hz, 2C), 111.4, 113.1 (t, J = 21.7 Hz), 117.1 (q, J = 289.9 Hz), 122.8 (q, J = 3.2 Hz), 126.3, 129.5 (t, J = 11.2 Hz), 141.0 (t, J = 2.4 Hz), 161.2 (dd, J = 243.7, 10.0 Hz, 2C), 168.0 (q, J = 35.1 Hz).

5-(3,5-Difluoro-4-methylphenyl)-1H-pyrrole-2-carboxylic acid (4)

Trifluoroethanone **3** (8.56 g, 29.6 mmol) and *n*-Bu₄NBr (~0.5 g) was added to a solution of NaOH (3.55 g, 88.8 mmol, 3 equiv) in a H₂O/THF mixture (89 + 25 mL). The resulting reaction mixture was refluxed for 12 h and cooled to r.t. The reaction mixture was extracted with Et₂O (2×100 mL) to remove unreacted SM. To the aqueous layer concentrated aqueous HCl solution (~8 mL, ~12 M) was added dropwise. The resulting precipitate was filtered off and washed with H₂O. M = 4.51 g. Yield = 64%.

¹H NMR: (DMSO, 400 MHz) δ = 2.12 (s, 3 H), 6.75 (dd, J = 3.8, 2.4 Hz, 1 H), 6.78 (dd, J = 3.9, 2.3 Hz, 1 H), 7.62 (d, J = 9.0 Hz, 2 H), 12.08 (s, 1 H), 12.48 (br. s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 6.8 (t, J = 2.9 Hz), 107.5 (dd, J = 19.8, 8.1 Hz, 2C), 108.9, 110.9 (t, J = 22.0 Hz), 116.3, 125.0, 131.5 (t, J = 11.0 Hz), 134.3 (t, J = 2.2 Hz), 161.3 (dd, J = 243.0, 10.3 Hz, 2C), 161.9.

2-(3-Fluoro-4-(trifluoromethyl)phenyl)-1H-pyrrole (5)

4-Bromo-2-fluoro-1-(trifluoromethyl)benzene (20.0 g, 82.3 mmol), pyrrole (17.3 mL, 248 mmol, 3 equiv), KOAc (24.24 g, 247 mmol, 3 equiv), and [PdCl(C₃H₅)₂] (302 mg) were dissolved in DMA (246 mL) under a constant flow of N₂. The reaction mixture was stirred at reflux for 8 h, then cooled to room temperature, poured into H₂O (1 L), and extracted with Et₂O (5 × 100 mL). The combined organic phases were evaporated in vacuo. The residue was extracted with DCM. The organic phase was dried over Na₂SO₄, filtered and evaporated. The residue was used without further purification. M = 20.35 g. Yield >100%.

¹H NMR: (CDCl₃, 400 MHz) δ = 6.33 (dd, J = 6.1, 2.6 Hz, 1 H), 6.63 – 6.67 (m, 1 H), 6.91 – 6.95 (m, 1 H), 7.29 – 7.36 (m, 2 H), 7.54 (t, J = 8.1 Hz, 1 H), 9.26 (br. s, 1 H).

2,2,2-Trifluoro-1-(5-(3-fluoro-4-(trifluoromethyl)phenyl)-1H-pyrrol-2-yl)ethanone (6)

Crude pyrrole **5** (20.35 g, 88.9 mmol) was dissolved in CH₂Cl₂ (90 mL) and pyridine (8.62 mL, 106.5 mmol, 1.2 equiv) was added followed by dropwise addition of TFAA (14.82 mL, 106.5 mmol, 1.2 equiv). After completion of the addition, the mixture was stirred for 1 h and the precipitate was filtered and washed with CH₂Cl₂. M = 11.27 g. Yield = 42% (over two steps).

¹H NMR: (DMSO, 400 MHz) δ = 7.10 (d, J = 4.3 Hz, 1 H), 7.26 (ddd, J = 6.2, 4.0, 2.0 Hz, 1 H), 7.80 (t, J = 8.1 Hz, 1 H), 7.98 (d, J = 8.3 Hz, 1 H), 8.16 (d, J = 12.6 Hz, 1 H), 13.11 (s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 112.2, 114.2 (d, J = 22.7 Hz), 116.2 (qd, J = 32.2, 12.4 Hz), 117.0 (d, J = 289.8 Hz), 122.3 (d, J = 2.2 Hz), 122.5 (d, J = 2.9 Hz), 122.6 (q, J = 271.5 Hz), 127.1, 127.6 (q, J = 2.9 Hz), 136.3 (d, J = 8.8 Hz), 139.9, 159.3 (d, J = 252.5 Hz), 168.6 (q, J = 34.4 Hz).

5-(3-Fluoro-4-(trifluoromethyl)phenyl)-1H-pyrrole-2-carboxylic acid (7)

Trifluoroethanone **6** (10.26 g, 31.6 mmol) was added to a solution of NaOH (6.31 g, 157.8 mmol, 5 equiv) in an H₂O/EtOH mixture (80 + 80 mL). The resulting reaction mixture was refluxed for 12 h and cooled to r.t. A concentrated aqueous HCl solution (~13 mL, ~12 M) was added dropwise. The resulting precipitate was filtered off and washed with H₂O.

M = 7.17 g. Yield = 83%.

¹H NMR: (DMSO, 400 MHz) δ = 6.80 – 6.84 (m, 1 H), 6.84 – 6.88 (m, 1 H), 7.70 (t, J =8.1 Hz, 1 H), 7.86 (d, J =8.3 Hz, 1 H), 8.05 (d, J =13.0 Hz, 1 H), 12.30 (s, 1 H), 12.62 (br. s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 110.4, 112.7 (d, J = 22.7 Hz), 114.2 (qd, J = 32.9, 12.4 Hz), 116.4, 121.0 (d, J = 2.9 Hz), 122.9 (q, J = 271.5 Hz), 126.1, 127.6 (q, J = 2.9 Hz), 133.5 (d, J = 2.2 Hz), 138.3 (d, J = 9.5 Hz), 159.4 (dq, J = 251.0, 2.2 Hz), 161.8.

1-(5-(3,5-Difluoro-4-(trifluoromethyl)phenyl)-1H-pyrrol-2-yl)-2,2,2-trifluoroethanone (9)

3,5-Difluoro-4-(trifluoromethyl)bromobenzene (20.0 g, 76.6 mmol), pyrrole (16.0 mL, 229 mmol, 3 equiv), KOAc (22.56 g, 230 mmol, 3 equiv), dppf (849 mg, 2 mol. %) and Pd(dba)₂ (881 mg, 2 mol. %) were dissolved in DMA (230 mL) under a constant flow of N₂. The reaction mixture was stirred at reflux for 8 h, then cooled to room temperature, poured into H₂O (1 L), and extracted with Et₂O (5 × 100 mL). The combined organic phases were evaporated in vacuo. The residue was extracted with DCM. The organic phase was dried over Na₂SO₄, filtered and evaporated. The residue was used without further purification. M = 30.71 g. Yield >100%.

Crude pyrrole **8** (30.71 g) was dissolved in DCM (124 mL) and pyridine (12.0 mL, 148 mmol, 1.2 equiv) was added followed by dropwise addition of TFAA (21.0 mL, 151 mmol, 1.2 equiv). After completion of the addition, the mixture was stirred for 1 h and washed with water. The organic layer was dried over Na₂SO₄, filtered and evaporated. The residue was purified using column chromatography (eluent: hexanes/EtOAc, 10:1). M = 2.76 g. Yield = 11% (over two steps).

¹H NMR: (DMSO, 400 MHz) δ = 7.20 (dd, J =4.2, 2.4 Hz, 1 H), 7.28 (dt, J = 4.1, 2.0 Hz, 1 H), 8.06 (d, J =11.7 Hz, 2 H), 13.14 (br. s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 105.0 (m), 110.4 (dd, J = 24.9, 2.9 Hz, 2C), 113.1, 116.8 (q, J = 289.8 Hz), 121.6 (q, J = 272.2 Hz), 122.5 (q, J = 2.9 Hz), 127.3, 136.7 (t, J = 12.1 Hz), 138.6, 159.4 (d, J = 255.4 Hz, 2C), 168.7 (q, J = 35.4 Hz).

5-(3,5-Difluoro-4-(trifluoromethyl)phenyl)-1H-pyrrole-2-carboxylic acid (10)

Trifluoroethanone **9** (1.12 g, 3.26 mmol) was added to a solution of NaOH (0.65 g, 16.3 mmol, 5 equiv) in a H₂O/EtOH mixture (16 + 16 mL). The resulting reaction mixture was refluxed for 12 h and cooled to r.t. A concentrated aqueous HCl solution (~1.4 mL, ~12 M) was added dropwise. The resulting precipitate was filtered off and washed with H₂O.

M = 893 mg. Yield = 94%.

¹H NMR: (DMSO, 400 MHz) δ = 6.82 (dd, J =3.8, 2.2 Hz, 1 H), 6.97 (dd, J =3.9, 2.6 Hz, 1 H), 7.92 (d, J =12.0 Hz, 2 H), 12.36 (s, 1 H), 12.74 (br. s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 103.1 (m), 108.8 (d, J = 24.9 Hz, 2C), 111.4, 116.3, 121.8 (q, J = 272.3 Hz), 126.7, 132.4, 138.6 (t, J = 12.1 Hz), 159.5 (d, J = 252.2 Hz, 2C), 161.7.

Ethyl thiazole-4-carboxylate (13)

To a solution of formamide (47 mL, 1.19 mol, 1.5 equiv) in 1,4-dioxane (318 mL), P₂S₅ (52.70 g, 0.237 mmol, 0.3 equiv) was added in small portions in the course of 0.5 h under vigorous stirring. The reaction mixture was stirred for 0.5 h, and ethyl bromopyruvate (154 g, 0.790 mol) was added dropwise. The reaction mixture was heated at reflux for 8 h, cooled to room temperature and a solution of K₂CO₃ (~100 g) in water (~500 mL) was carefully added to the reaction mixture. The resulted solution was extracted with DCM (3×100 mL). The combined organic phases were dried over anhydrous Na₂SO₄, filtered, evaporated, and the residue was distilled at reduced pressure. bp = 120 – 130 °C (10 torr). M = 108.3 g. Yield = 87%.

¹H NMR: (CDCl₃, 400 MHz) δ = 1.40 (t, J =7.2 Hz, 3 H), 4.42 (q, J =7.1 Hz, 2 H), 8.24 (d, J =2.1 Hz, 1 H), 8.87 (d, J =2.1 Hz, 1 H).

¹³C NMR (CDCl₃, 100 MHz): δ = 14.4, 61.7, 127.3, 148.2, 153.6, 161.3.

Thiazol-4-ylmethanol (14)

A solution of **13** (108.25 g, 0.689 mol) in THF (344 mL) was added dropwise to a suspension of LiAlH₄ (26.3 g, 0.692 mmol) in THF (344 mL) at 0 °C. The reaction mixture was stirred for 30 min at 0 °C (at this point TLC indicated consumption of the SM). It was then quenched by successive addition of EtOAc (50 mL), water (26 mL), 10% NaOH (26 mL) solution, and water (78 mL) (the temperature should not exceed 0 °C). The precipitate was filtered and washed several times with THF. The filtrate was evaporated to give **8**, which was used without purification. M = 43.25 g. Yield = 54%.

¹H NMR: (CDCl₃, 400 MHz) δ = 4.13 (br. s, 1 H), 4.79 (s, 2 H), 7.25 (s, 1 H), 8.76 (s, 1 H).

¹³C NMR (CDCl₃, 100 MHz): δ = 60.3, 114.8, 153.6, 157.4

4-(((tert-Butyldimethylsilyl)oxy)methyl)thiazole (15)

Alcohol **14** (25.4 g, 0.221 mol) was dissolved in DMF (220 mL), and imidazole (22.5 g, 0.330 mol, 1.5 equiv) was added in one portion, followed by portionwise addition of TBSCl (50.0 g, 0.332 mol, 1.5 equiv). The reaction mixture was stirred overnight at 50–60 °C, cooled to rt, diluted with water (0.5 L), and extracted with hexane (3 × 100 mL). The combined organic phases were dried over anhydrous Na₂SO₄, filtered, and evaporated to give an oil, which was purified by distillation at reduced pressure. bp = 90–110 °C, 1–2 Torr. M = 36.6 g. Yield = 72%.

¹H NMR: (CDCl₃, 400 MHz) δ = 0.12 (s, 6 H), 0.94 (s, 9 H), 4.92 (s, 2 H), 7.24 (s, 1 H), 8.75 (d, J =1.8 Hz, 1 H).

¹³C NMR: (CDCl₃, 100 MHz): δ = -5.2 (2C), 18.5, 26.0 (3C), 62.4, 113.6, 152.9, 158.4.

2-(Thiazol-4-yl)propan-2-ol (16)

Methyl Iodide (140 mL, 2.25 mol, 5.0 equiv) was dissolved in Et₂O (450 mL) and added dropwise to Mg turnings (54.3 g, 2.25 mol, 5.0 equiv) under a constant flow of nitrogen. When all magnesium dissolves ester **13** (70.24 g, 447 mmol) in Et₂O (450 mL) was added dropwise. The reaction mixture was left overnight and resulted suspension was carefully (*very exothermic + gas evolution!*) was poured into saturated aqueous NH₄Cl (~2L). The resulted solution was extracted with Et₂O (3×200 mL). The combined organic layers were dried over Na₂SO₄ and evaporated to give the pure compound. M = 48.26 g. Yield = 75%.

¹H NMR: (CDCl₃, 400 MHz) δ = 1.61 (s, 6 H), 3.27 (br. s, 1 H), 7.17 (d, J =2.0 Hz, 1 H), 8.73 (d, J =1.8 Hz, 1 H).

¹³C NMR: (CDCl₃, 100 MHz) δ = 30.2 (2C), 71.2, 77.2, 111.6, 152.9, 165.0.

4-((tert-Butyldimethylsilyl)oxy)propan-2-ylthiazole (17)

Alcohol **16** (48.26 g, 337 mmol) was dissolved in DMF (340 mL), and imidazole (68.8 g, 1.01 mol, 3 equiv) was added in one portion, followed by portion wise addition of TBSCl (101.6 g, 0.674 mol, 2 equiv). The reaction mixture was stirred for two days at ~80 °C, cooled to rt, diluted with water (1 L), and extracted with hexane (3×200 mL). The combined organic phases were dried over anhydrous Na₂SO₄, filtered, and evaporated to give an oil, which was purified by distillation at reduced pressure. bp = 100–110 °C, 1–2 Torr. M = 56.3 g. Yield = 65%.

¹H NMR: (CDCl₃, 400 MHz) δ = 0.08 (s, 6 H), 0.93 (s, 9 H), 1.63 (s, 6 H), 7.25 (d, J =2.1 Hz, 1 H), 8.73 (d, J =2.2 Hz, 1 H).

¹³C NMR: (CDCl₃, 100 MHz) δ = -2.1 (2C), 18.3, 26.0 (3C), 31.3 (2C), 74.9, 77.2, 112.3, 152.2, 166.3.

Diethyl 2-chloro-3-oxosuccinate (18)

NaO^tBu (96.1 g, 1.0 mol) was dissolved in THF (1 L), and the solution was cooled to 0 °C. At this temperature with vigorous mechanical stirring, a mixture of ethyl chloroacetate (107 mL, 1.0 mol) and diethyl oxalate (136 mL, 1.0 mol) was added dropwise. The mixture was left overnight, and aqueous HCl (90 ml + 400 ml H₂O) was added. The resulted solution was extracted with CH₂Cl₂ (3×200 mL). The combined organic layers were dried over Na₂SO₄, filtered and evaporated. The residue was distilled at reduced pressure (bp = 110–140 °C, 10 torr). M = 157.6 g. Yield = 71%.

¹H NMR: (CDCl₃, 400 MHz) δ = 1.31 (t, J =7.2 Hz, 3 H), 1.39 (t, J =7.2 Hz, 3 H), 4.31 (q, J =7.2 Hz, 2 H), 4.39 (q, J =7.2 Hz, 2 H), 5.46 (s, 1 H).

Diethyl thiazole-4,5-dicarboxylate (19)

To a solution of formamide (21 mL, 529 mmol, 1.5 equiv) in 1,4-dioxane (150 mL), P₂S₅ (23.70 g, 0.107 mmol, 0.3 equiv) was added in small portions in the course of 0.5 h under vigorous stirring. The reaction mixture was stirred for 0.5 h, and **18** (79.0 g, 0.355 mol) was added dropwise. The reaction mixture was heated at reflux for 8 h, cooled to room temperature and a solution of K₂CO₃ (~100 g) in water (~500 mL) was carefully added to the reaction mixture. The resulted solution was extracted with DCM (3×100 mL). The combined organic phases were dried over anhydrous Na₂SO₄, filtered, evaporated, and the residue was purified using column chromatography. Eluent: hexane/EtOAc (10:1, 3:1, 1:1). M = 55.1 g. Yield = 68%.

¹H NMR: (CDCl₃, 400 MHz) δ = 1.37 (t, *J*=7.2 Hz, 3 H), 1.41 (t, *J*=7.2 Hz, 3 H), 4.38 (q, *J*=7.1 Hz, 2 H), 4.45 (q, *J*=7.2 Hz, 2 H), 8.85 (s, 1 H).

¹³C NMR: (CDCl₃, 100 MHz) δ = 14.2 (2C), 62.5, 62.6, 130.6, 150.0, 155.6, 160.3, 162.5.

Thiazole-4,5-diylldimethanol (20)

A solution of diester **19** (110.25 g, 0.481 mol) in THF (480 mL) was added dropwise to a suspension of LiAlH₄ (36.5 g, 0.961 mmol, 2.0 equiv) in THF (480 mL) at 0 °C. The reaction mixture was stirred for 60 min at 0 °C. It was then quenched by successive addition of EtOAc (100 mL), water (37 mL), 10% NaOH (37 mL) solution, and water (74 mL) (the temperature should not exceed 0 °C). The precipitate was filtered and washed several times with THF. The filtrate was evaporated to give **20**, which was used without purification. M = 33.09 g. Yield = 47%.

¹H NMR: (DMSO, 400 MHz) δ = 4.53 (d, *J* = 5.6 Hz, 2H), 4.74 (d, *J* = 5.6 Hz, 2H), 5.09 (t, *J* = 5.6 Hz, 1H), 5.55 (t, *J* = 5.6 Hz, 1H), 8.88 (s, 1H).

¹³C NMR: (DMSO, 100 MHz) δ = 55.1, 57.3, 136.6, 151.2, 151.5.

4,5-Bis(((tert-butyldimethylsilyl)oxy)methyl)thiazole (21)

Alcohol **20** (33.09 g, 0.228 mol) was dissolved in DMF (230 mL), and imidazole (46.6 g, 0.684 mol, 3.0 equiv) was added in one portion, followed by portion wise addition of TBSCl (103.0 g, 0.683 mol, 3.0 equiv). The reaction mixture was stirred overnight at 60–80 °C, cooled to rt, diluted with water (0.5 L), and extracted with hexane (3 × 100 mL). The combined organic phases were dried over Na₂SO₄, filtered, and evaporated to give brown oil, which was purified by column chromatography. Eluent: hexanes/EtOAc (10:1). After chromatography, the title compound was dried at reduced pressure (80 °C, 1–2 Torr). M = 60.8 g. Yield = 71%.

¹H NMR: (CDCl₃, 400 MHz) δ = 0.09 (s, 6 H), 0.11 (s, 6 H), 0.92 (s, 9 H), 0.93 (s, 9 H), 4.87 (s, 2 H), 5.02 (s, 2 H), 8.59 (s, 1 H).

¹³C NMR: (CDCl₃, 100 MHz) δ = -5.3, 18.4, 18.5, 25.9 (3C), 26.0 (3C), 58.5, 61.4, 137.1, 149.7, 150.5.

Allyl allyl(2-(4-(((tert-butyldimethylsilyl)oxy)methyl)thiazol-2-yl)-2-(1,1-dimethylethyl-sulfinamido)ethyl)carbamate (24)

Thiazole **15** (14.90 g, 64.95 mmol, 1.2 equiv) was dissolved in THF (65 mL) and cooled to -78°C . At this temperature, *n*-BuLi (2.5 M, 28 mL, 70.0 mmol, 1.3 equiv) was added dropwise under a nitrogen atmosphere. The reaction mixture was stirred for 20 min at -78°C , and *S*-(*E*)-allyl allyl(2-((*tert*-butylsulfinyl)imino)ethyl)carbamate **22** (15.50 g, 54.1 mmol) was added dropwise as a solution in THF (54 mL). The reaction mixture was slowly (~1 h) warmed to 0°C and poured into water (0.5 L). The biphasic mixture was extracted with DCM (3×100 mL). The combined organic phases were dried over Na_2SO_4 , filtered, and evaporated to give brown oil which was purified by column chromatography. Eluent: hexanes/EtOAc (3:1, 1:1).

24A: M = 22.40 g. Yield = 80%. The second enantiomer was prepared from *R*-imine **22**:

24B: M = 22.45 g. Yield = 83%

^1H NMR: (CDCl_3 , 400 MHz) δ = 0.09 (s, 6 H), 0.92 (s, 9 H), 1.27 (s, 9 H), 3.56 (dd, J =14.6, 2.9 Hz, 1 H), 3.80 (dd, J =16.3, 5.4 Hz, 1 H), 3.85 – 4.00 (m, 2 H), 4.52 – 4.67 (m, 2 H), 4.79 (d, J =0.6 Hz, 2 H), 4.93 (dt, J =9.2, 3.9 Hz, 1 H), 5.09 – 5.22 (m, 3 H), 5.28 (d, J =17.4 Hz, 1 H), 5.59 (d, J =2.9 Hz, 1 H), 5.73 – 5.85 (m, 1 H), 5.85 – 5.96 (m, 1 H), 7.09 (s, 1 H).

^{13}C NMR (CDCl_3 , 100 MHz): δ = -5.2 (2C), 18.5, 22.9 (3C), 26.0 (3C), 50.5, 52.2, 56.3, 57.9, 62.3, 66.8, 114.2, 117.1, 117.5, 132.7, 133.1, 158.1, 158.6, 173.2.

Allyl allyl(2-amino-2-(4-(hydroxymethyl)thiazol-2-yl)ethyl)carbamate (25)

A 1 M HCl-MeOH solution was prepared by dropwise addition of AcCl to a MeOH. The resulting solution (~250 mL) was cooled to an ambient temperature and added to a flask containing protected compound **24A** (22.40 g, 43.4 mmol). After dissolution, the reaction mixture was stirred for 1 h, evaporated, dissolved in DCM (100 mL), and washed with 10% aqueous K_2CO_3 (200 mL). The organic layer was separated and the aqueous layer was extracted with DCM (2×100 mL). The combined organic layers were dried over Na_2SO_4 , filtered, and evaporated and loaded on silica. Eluting with DCM/MeOH (20:1, 10:1) provided pure amine as yellow oil.

25A: M = 5.33 g. Yield = 41%. The second enantiomer was prepared from **24B**:

25B: M = 7.52 g. Yield = 58%.

^1H NMR: (CDCl_3 , 400 MHz) δ = 2.73 (br. s, 3 H), 3.47 – 3.69 (m, 2 H), 3.71 – 3.93 (m, 2 H), 4.44 (t, J =5.9 Hz, 1 H), 4.55 (br. s, 2 H), 4.67 (s, 2 H), 5.10 (d, J =7.8 Hz, 2 H), 5.17 (dd, J =10.5, 1.2 Hz, 1 H), 5.26 (dd, J =17.2, 1.5 Hz, 1 H), 5.63 – 5.79 (m, 1 H), 5.88 (dddd, J =16.8, 10.9, 5.5, 5.3 Hz, 1 H), 7.09 (s, 1 H).

^{13}C NMR (CDCl_3 , 100 MHz): δ = (50.7, 53.2), (53.3, 54.1), (60.5, 60.7), 66.4, (114.6, 114.8), (117.0, 117.5, 117.9), (132.8, 133.2), 153.4, (156.2, 156.9), 156.8, (175.1, 175.6).

Allyl (2-(4-(((tert-butyldimethylsilyl)oxy)methyl)thiazol-2-yl)-2-(1,1-dimethylethylsulfonamido)ethyl)(methyl)carbamate (26)

Thiazole **15** (14.30 g, 62.4 mmol, 1.5 equiv) was dissolved in THF (41 mL) and cooled to -78°C . At this temperature, *n*-BuLi (2.5 M, 25 mL, 62.5 mmol, 1.5 equiv) was added dropwise under a nitrogen atmosphere. The reaction mixture was stirred for 20 min at -78°C , and *S*-(*E*)-allyl (2-(((*tert*-butylsulfinyl)imino)ethyl)(methyl)carbamate **23** (10.81 g, 41.6 mmol) was added dropwise as a solution in THF (41 mL). The reaction mixture was slowly (~ 1 h) warmed to 0°C and poured into water (0.5 L). The biphasic mixture was extracted with DCM (3×100 mL). The combined organic phases were dried over Na_2SO_4 , filtered, and evaporated to give a brown oil which was purified by column chromatography. Eluent: hexanes/EtOAc (10:1, 1:1, 0:1).

26A: M = 15.45 g. Yield = 76%. The second enantiomer was prepared from *R*-imine **23**:

26B: M = 25.95 g. Yield > 100%

^1H NMR: (CDCl_3 , 400 MHz) δ = 0.09 (s, 6 H), 0.91 (s, 9 H), 1.26 (s, 9 H), 2.96 (s, 3 H), 3.52 (d, J =14.4 Hz, 1 H), 4.00 (dd, J =14.1, 10.0 Hz, 1 H), 4.51 – 4.67 (m, 2 H), 4.80 (s, 2 H), 4.88 – 4.98 (m, 1 H), 5.19 (d, J =10.4 Hz, 1 H), 5.29 (d, J =17.2 Hz, 1 H), 5.37 – 5.45 (m, 1 H), 5.84 – 6.00 (m, 1 H), 7.09 (s, 1 H).

^{13}C NMR (CDCl_3 , 100 MHz): δ = -5.2 (2C), 18.5, 22.9 (3C), 26.0 (3C), 35.1, 54.4, 56.2, 57.3, 62.3, 66.8, 114.2, 117.6, 132.9, 158.2, 158.7, 173.3

Allyl (2-amino-2-(4-(hydroxymethyl)thiazol-2-yl)ethyl)(methyl)carbamate (27)

A 1 M HCl-MeOH solution was prepared by dropwise addition of AcCl to a MeOH. The resulting solution (~ 150 mL) was cooled to ambient temperature and added to a flask containing protected compound **26A** (15.45 g, 31.5 mmol). After dissolution, the reaction mixture was stirred for 1 h, evaporated, dissolved in DCM (100 mL), and washed with 10% aqueous K_2CO_3 (200 mL). The organic layer was separated, and the aqueous layer was extracted with DCM (2×100 mL). The combined organic layers were dried over Na_2SO_4 , filtered, and evaporated and loaded on silica. Eluting with DCM/MeOH (10:1, 1:1) provided pure amine as a yellow oil.

27A: M = 6.15 g. Yield = 72%. The second enantiomer was prepared from **26B**:

27B: M = 3.81 g. Yield = 32% (over two steps).

^1H NMR: (CDCl_3 , 400 MHz) δ = 2.83 (br. s., 3 H), 2.89 (br. s., 3 H), 3.43 – 3.72 (m, 2 H), 4.35 – 4.45 (m, 1 H), 4.45 – 4.54 (m, 2 H), 4.64 (s, 2 H), 5.14 (d, J =9.9 Hz, 1 H), 5.23 (d, J =16.6 Hz, 1 H), 5.83 (br. s., 1 H), 7.08 (s, 1 H).

^{13}C NMR (CDCl_3 , 100 MHz): δ = (35.3, 35.6), (52.7, 52.8), (55.6, 56.0), 60.5, 66.3, 114.8, (117.4, 117.8), 132.8, (156.3, 157.0), 156.9, (174.9, 175.4).

Allyl allyl(2-(4,5-bis(((tert-butyldimethylsilyl)oxy)methyl)thiazol-2-yl)-2-(1,1-dimethylethyl-sulfinamido)ethyl)carbamate (28)

Thiazole **21** (28.3 g, 75.7 mmol, 1.5 equiv) was dissolved in THF (76 mL) and cooled to -78 °C. At this temperature, *n*-BuLi (2.5 M, 32 mL, 80 mmol, 1.6 equiv) was added dropwise under a nitrogen atmosphere. The reaction mixture was stirred for 5–10 min at -78 °C, and *R*-(*E*)-allyl allyl(2-((*tert*-butylsulfinyl)imino)ethyl)carbamate **22** (14.45 g, 50.5 mmol) was added dropwise as a solution in THF (50 mL). The reaction mixture was slowly (~1 h) warmed to 0 °C and poured into water (0.5 L). The biphasic mixture was extracted with DCM (3 × 100 mL). The combined organic phases were dried over Na₂SO₄, filtered, and evaporated to give brown oil which was purified by column chromatography. Eluent: hexanes/EtOAc (3:1, 1:1).

28B: M = 29.08 g. Yield = 87%. The second enantiomer was prepared from *S*-imine **22**:

28A: M = 24.46 g. Yield = 73%.

¹H NMR: (CDCl₃, 400 MHz) δ = 0.08 (s, 12 H), 0.90 (d, *J*=1.7 Hz, 18 H), 1.27 (s, 9 H), 3.53 (dd, *J*=14.7, 2.9 Hz, 1 H), 3.81 (dd, *J*=16.3, 5.4 Hz, 1 H), 3.88 – 4.04 (m, 2 H), 4.61 (ddd, *J*=27.8, 13.4, 5.9 Hz, 2 H), 4.76 (s, 2 H), 4.87 (dt, *J*=9.3, 3.8 Hz, 1 H), 4.93 (s, 2 H), 5.11 – 5.23 (m, 3 H), 5.30 (d, *J*=17.2 Hz, 1 H), 5.56 (d, *J*=2.9 Hz, 1 H), 5.74 – 5.86 (m, 1 H), 5.86 – 5.99 (m, 1 H).

¹³C NMR: (CDCl₃, 100 MHz) δ = -5.3 (2C), -5.2 (2C), 18.3, 18.5, 23.0 (3C), 25.9 (3C), 26.0 (3C), 50.5, 52.2, 56.3, 58.0, 58.5, 61.3, 66.8, 117.2, 117.6, 132.8, 133.1, 137.3, 149.6, 158.6, 170.2.

Allyl allyl(2-amino-2-(4,5-bis(hydroxymethyl)thiazol-2-yl)ethyl)carbamate (29)

A 1 M HCl MeOH solution was prepared by dropwise addition of AcCl to a MeOH. The resulting solution (~300 mL) was cooled to ambient temperature and added to a flask containing protected compound **29A** (24.46 g, 37.1 mmol). After dissolution, the reaction mixture was stirred for 1 h. HCl was neutralized by addition of excess of solid NaOH (~14 g). The reaction mixture was stirred for 10 minutes and dry loaded on silica. Eluting with EtOAc, followed by DCM/MeOH (5:1) provided pure amine as a yellow oil. TLC CHCl₃/MeOH saturated with NH₃ (5:1).

29A: M = 8.97 g. Yield = 74%. The second enantiomer was prepared from **28B**:

29B: M = 2.57 g. Yield = 30%.

¹H NMR: (CDCl₃, 400 MHz) δ = 3.45 – 3.83 (m, 7 H), 3.83 – 3.93 (m, 1 H), 4.40 (t, *J*=6.7 Hz, 1 H), 4.56 (br. s, 2 H), 4.60 (br. s, 2 H), 4.72 (s, 2 H), 5.06 – 5.16 (m, 2 H), 5.20 (dq, *J*=10.5, 1.2 Hz, 1 H), 5.28 (dq, *J*=17.2, 1.5 Hz, 1 H), 5.74 (td, *J*=10.7, 5.3 Hz, 1 H), 5.89 (ddd, *J*=16.5, 11.1, 5.3 Hz, 1 H).

¹³C NMR: (CDCl₃, 100 MHz) δ = 50.7, 52.8, (53.2, 54.0), 55.6, 58.1, 66.4, (117.1, 117.6, 118.0), 132.6, 133.1, 135.2, 151.7, (156.1, 156.9), 172.4.

Allyl allyl(2-(4-(2-((*tert*-butyldimethylsilyl)oxy)propan-2-yl)thiazol-2-yl)-2-(1,1-dimethyl-ethylsulfonamido)ethyl)carbamate (30)

Thiazole **17** (14.40 g, 55.9 mmol, 1.2 equiv) was dissolved in THF (56 mL) and cooled to -78°C . At this temperature, *n*-BuLi (2.5 M, 23 mL, 57.5 mmol, 1.2 equiv) was added dropwise under a nitrogen atmosphere. The reaction mixture was stirred for 5–10 min at -78°C , and *S*-(*E*)-allyl allyl(2-((*tert*-butylsulfinyl)imino)ethyl)carbamate **22** (13.30 g, 46.4 mmol) was added dropwise as a solution in THF (47 mL). The reaction mixture was slowly (~1 h) warmed to 0°C and poured into water (0.5 L). The biphasic mixture was extracted with DCM (3×100 mL). The combined organic phases were dried over Na_2SO_4 , filtered, and evaporated to give brown oil which was purified by column chromatography. Eluent: hexanes/EtOAc (3:1, 1:1).

30A: M = 27.51 g. Yield >100%. The second enantiomer was prepared from *R*-imine **22**:

30B: M = 30.90 g. Yield = 99%.

^1H NMR: (CDCl_3 , 400 MHz) δ = 0.06 (s, 6 H), 0.91 (s, 9 H), 1.28 (s, 9 H), 1.57 (d, $J=3.8$ Hz, 6 H), 3.71 – 3.99 (m, 2 H), 4.52 – 4.67 (m, 3 H), 4.91 – 4.96 (m, 1 H), 5.07 – 5.23 (m, 4 H), 5.29 (d, $J=16.9$ Hz, 1 H), 5.57 (d, $J=2.9$ Hz, 1 H), 5.72 – 5.85 (m, 1 H), 5.85 – 5.98 (m, 1 H), 7.09 (s, 1 H).

^{13}C NMR: (CDCl_3 , 100 MHz) δ = -2.3 (2C), 18.1, 22.8, 25.9 (3C), (30.96, 31.00) (2C), 50.4, 52.1, 56.2, 57.7, 66.6, 74.4, 112.3, 117.1, 117.4, 132.6, 133.0, 158.3, 166.0, 171.8

Allyl allyl(2-amino-2-(4-(2-hydroxypropan-2-yl)thiazol-2-yl)ethyl)carbamate (31)

30A (9.04 g, 16.6 mmol) was dissolved in 1,4-dioxane (90 mL) and conc. aqueous HCl (9.0 mL, ~108 mmol) was added. The reaction mixture was stirred for 10 minutes, and neutralized with a solution of NaOH (9.0 g, 225 mmol) in water (200 mL). The resulted solution was extracted with DCM (3×100 mL). The combined organic phases were dried over Na_2SO_4 , filtered, and evaporated to give brown oil, which was purified by column chromatography. Eluent: EtOAc, DCM/MeOH (10:1).

31A: M = 2.38 g. Yield = 48% (over two steps). The second enantiomer was prepared from **30B**:

31B: M = 1.11 g. Yield = 52%.

^1H NMR: (CDCl_3 , 400 MHz) δ = 1.54 (s, 6 H), 1.86 (br. s, 2 H), 3.08 (br. s, 1 H), 3.48 – 3.94 (m, 4 H), 4.39 – 4.47 (m, 1 H), 4.50 – 4.60 (m, 2 H), 5.00 – 5.14 (m, 2 H), 5.17 (dd, $J=10.5, 0.7$ Hz, 1 H), 5.27 (d, $J=17.1$ Hz, 1 H), 5.63 – 5.80 (m, 1 H), 5.82 – 5.94 (m, 1 H), 6.99 (s, 1 H).

^{13}C NMR: (CDCl_3 , 100 MHz) δ = 29.9 (2C), (50.4, 50.5), 52.8, (53.1, 53.8), 66.0, 70.8, 111.2, 116.6, 117.1, 117.5, 132.5, 133.1, (155.8, 156.6), 164.1, (173.9, 174.4).

Allyl allyl(2-amino-2-(4-(2-methoxypropan-2-yl)thiazol-2-yl)ethyl)carbamate (32B)

A 1 M HCl MeOH solution was prepared by dropwise addition of AcCl to a MeOH. The resulting solution (~200 mL) was cooled to an ambient temperature and added to a flask containing protected compound **30B** (24.40 g, 44.9 mmol). After dissolution, the reaction mixture was stirred for 1 h, evaporated, dissolved in DCM (100 mL), and washed with 10% aqueous K₂CO₃ (200 mL). The organic layer was separated and the aqueous layer was extracted with DCM (2×100 mL). The combined organic layers were dried over Na₂SO₄, filtered, and evaporated and loaded on silica. Eluting with DCM/MeOH (20:1, 10:1) provided pure amine as yellow oil. M = 5.58 g. Yield = 37%.

¹H NMR: (CDCl₃, 400 MHz) δ = 1.54 (s, 6 H), 2.57 (br. s, 2H), 3.10 (s, 3 H), 3.51 – 3.93 (m, 4 H), 4.45 – 4.61 (m, 3 H), 5.01 – 5.15 (m, 2 H), 5.18 (d, *J*=10.3 Hz, 1 H), 5.22 – 5.33 (m, 1 H), 5.64 – 5.79 (m, 1 H), 5.83 – 5.96 (m, 1 H), 7.06 (s, 1 H).

¹³C NMR: (CDCl₃, 100 MHz) δ = (25.9, 26.5) (2C), 50.2, 50.4, 53.0, (53.2, 53.8), 65.9, 75.3, 113.8, 116.5, 117.1, 117.4, 132.6, 133.1, (155.8, 156.6), 160.9, (174.0, 174.7).

Ethyl oxazole-5-carboxylate (33)

To a stirred solution of TosMIC (50.0 g, 256 mmol) in CH₂Cl₂ (255 mL) was added ethyl glyoxylate (~50% in toluene, 56 mL, 283 mmol, 1.1 equiv). The solution was cooled to 0 °C, and at this temperature, DBU (38 mL, 254 mmol) was added dropwise. The reaction mixture was allowed to warm to ambient temperature and stirred for 8 hours. The reaction mixture was washed with water (~300 mL), organic layers were dried over Na₂SO₄ and evaporated. The residue was distilled at reduced pressure. bp = 98 °C (25 mbar). M = 18.3 g. Yield = 51%.

¹H NMR: (CDCl₃, 400 MHz) δ = 1.35 (t, *J*=7.2 Hz, 3 H), 4.35 (q, *J*=7.1 Hz, 2 H), 7.73 (s, 1 H), 7.99 (s, 1 H).

¹³C NMR (CDCl₃, 100 MHz): δ = 14.2, 61.7, 133.3, 142.9, 153.3, 157.6.

Oxazol-5-ylmethanol (34)

A solution of **33** (29.13 g, 0.207 mol) in THF (206 mL) was added dropwise to a suspension of LiAlH₄ (7.85 g, 0.207 mmol) in THF (206 mL) at 0 °C. The reaction mixture was stirred for 30 min at 0 °C (at this point TLC indicated consumption of the SM) and quenched by successive addition of water (8 mL), 10% NaOH (8 mL) solution, and water (24 mL). The temperature should not exceed 0 °C. The precipitate was filtered and washed several times with THF. The filtrate was evaporated to give **34**, which was used without purification. M = 17.7 g. Yield = 86%.

¹H NMR: (CDCl₃, 400 MHz) δ = 2.89 (br. s, 1 H), 4.68 (s, 2 H), 7.00 (s, 1 H), 7.85 (s, 1 H).

¹³C NMR (CDCl₃, 100 MHz): δ = 54.5, 123.5, 151.3, 151.8.

5-(((tert-Butyldimethylsilyl)oxy)methyl)oxazole (35)

Alcohol **34** (20.77 g, 0.210 mol) was dissolved in DMF (211 mL), and imidazole (21.4 g, 0.314 mol, 1.5 equiv) was added in one portion, followed by portionwise addition of TBSCl (47.4 g, 0.315 mol, 1.5 equiv). The reaction mixture was stirred overnight at 50–60 °C, cooled to room temperature, diluted with water (0.5 L), and extracted with hexane (3×100 mL). The combined organic phases were dried over anhydrous Na₂SO₄ and evaporated to give an oil, which was purified by distillation at reduced pressure (bp = 115–120 °C, 26 mbar). 65–70 °C, 1 mbar). M = 30.79 g. Yield = 69%.

¹H NMR: (CDCl₃, 400 MHz) δ = 0.09 (s, 6 H), 0.89 (s, 9 H), 4.69 (s, 2 H), 6.96 (s, 1 H), 7.83 (s, 1 H).

¹³C NMR (CDCl₃, 100 MHz): δ = –5.2 (2C), 18.4, 25.9 (3C), 56.0, 77.2, 123.8, 151.0.

Allyl allyl(2-amino-2-(5-(hydroxymethyl)oxazol-2-yl)ethyl)carbamate (37)

To a solution of oxazole **35** (8.10 g, 38.0 mmol) in THF (38 mL) at rt under nitrogen was added BH₃*Me₂S (3.97 mL, 47.5 mmol, 1.1 equiv). After 30 min, the solution was cooled to –78 °C, and n-BuLi (19 mL, 2.5 M in hexane, 1.2 equiv) was added dropwise. After 10 min, *S*-(*E*)-allyl allyl(2-((*tert*-butylsulfinyl)imino)ethyl)carbamate **22** (10.89 g, 38.0 mmol) was added dropwise as a solution in THF (38 mL). The reaction mixture was slowly (~1 h) warmed to rt, and 20 mL of acetic acid in ethanol (100 mL) was added dropwise. The mixture was stirred overnight at rt to cleave the borane complex. Then more ethanol (100 mL) was added followed by aqueous HCl (50 mL). The reaction mixture was stirred for 2 h, evaporated, dissolved in water (100 mL), and extracted with DCM (2×100 mL). The aqueous layer was basified with an excess of solid K₂CO₃. The resulted solution was extracted with DCM (5×100 mL). The combined organic layers were dried over Na₂SO₄, filtered, and evaporated and loaded on silica. Eluting with DCM/MeOH (10:1) provided pure amine as a yellow oil. **37A**: M = 5.17 g. Yield = 48% (over two steps).

The second enantiomer was prepared from *R*-imine **22**: **37B** M = 2.85 g. Yield = 45% (over two steps).

¹H NMR: (CDCl₃, 400 MHz) δ = 2.67 (br. s, 3 H), 3.57 (d, *J*=6.7 Hz, 2 H), 3.72–3.96 (m, 2 H), 4.26 (q, *J*=6.0 Hz, 1 H), 4.54 (d, *J*=5.1 Hz, 2 H), 4.57 (s, 2 H), 5.04–5.15 (m, 2 H), 5.17 (dd, *J*=10.5, 1.0 Hz, 1 H), 5.26 (dd, *J*=17.2, 1.3 Hz, 1 H), 5.66–5.79 (m, 1 H), 5.81–5.93 (m, 1 H), 6.87 (s, 1 H).

¹³C NMR (CDCl₃, 100 MHz): δ = 49.1, 50.7, (51.5, 52.1), 54.7, (66.4, 66.5), 117.1, 117.5, 117.9, 124.2, 132.7, 133.2, 151.8, 165.0 (br.).

General Procedure for Amide Coupling

DIPEA (1 equiv) was added to an appropriate acid (**4**, **7**, **10**, **11**, **12**; 1 equiv) followed by DMF (10 mL per 1 g of acid) and then HBTU (1 equiv). The resulting solution was stirred for 5 min and added to a solution of appropriate amine (**25**, **27**, **29**, **31**, **32**, **37**; **38** 1 equiv) in DMF (10 mL per 1 g of amine) in several portions. The reaction mixture was stirred overnight; DMF was evaporated, and the residue was dissolved in DCM (50 mL) and

successively washed with 5% aqueous NaOH and 10% tartaric acid or citric acid aqueous solutions (50 mL). The organic layer was dried over Na₂SO₄, filtered, evaporated, and dry loaded on silica. Eluting with hexanes/EtOAc (1:1, then pure EtOAc) gave the target compounds. The products were used in the next step without analysis.

General Procedure for Deprotection

To a solution containing protected compound (5 mmol) and N,N'-dimethyl barbituric acid (NDMBA, 15 mmol, 3 equiv) in MeOH (50 mL), PPh₃ (10 mol %) was added under a nitrogen atmosphere followed by Pd(dba)₂ (5 mol %). The mixture was stirred for 1 day under reflux. After cooling, 50 mL of DCM was added, and the organic phase was shaken with 10% aqueous K₂CO₃ (50 mL) to remove the unreacted NDMBA. The organic layer was separated, and the aqueous layer was extracted with DCM/EtOH (~4:1, (2–4) × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated. Purification by flash chromatography afforded amine as a slightly brown or yellowish solid.

N-(2-Amino-1-(5-(hydroxymethyl)thiazol-2-yl)ethyl)-5-(4-(trifluoromethyl)phenyl)-1H-pyrrole-2-carboxamide (**47** and **48**)

Compounds **47** and **48** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **38** and acid **12**. Compounds were purified using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1).

47: M = 515 mg. Yield = 52% (over two steps).

rt = 1.256 min. Purity = 96%. LC–MS: m/z [M+H]⁺ = 411 Da.

48: M = 525 mg. Yield = 47% (over two steps).

rt = 1.183 min. Purity = 100%. LC–MS: m/z [M+H]⁺ = 411 Da.

m.p. = 150–155 °C.

¹H NMR (DMSO-*d*₆, 400 MHz): δ = 1.74 (br. s, 2 H), 3.00 (dd, *J*=13.2, 7.9 Hz, 1 H), 3.13 (dd, *J*=13.2, 5.3 Hz, 1 H), 4.60 (s, 2 H), 5.19 (dd, *J*=13.0, 7.5 Hz, 1 H), 5.47 (br. s, 1 H), 6.77 (d, *J*=3.8 Hz, 1 H), 7.03 (d, *J*=3.8 Hz, 1 H), 7.54 (s, 1 H), 7.70 (d, *J*=8.3 Hz, 2 H), 8.02 (d, *J*=8.1 Hz, 2 H), 8.61 (d, *J*=7.8 Hz, 1 H), 12.00 (br. s, 1 H).

¹³C NMR (DMSO-*d*₆, 100 MHz): δ = 45.5, 54.4, 55.8, 108.8, 113.0, 124.4 (q, *J* = 271.5 Hz), 125.0, 125.6 (q, *J* = 3.7 Hz), 126.6 (q, *J* = 31.5 Hz), 128.3, 133.3, 135.6, 139.0, 140.1, 160.4, 171.9.

HRMS (ESI): m/z calcd for C₁₈H₁₈F₃N₄O₂S [M+H]⁺ 411.1097, found 411.1095.

N-(2-Amino-1-(5-(hydroxymethyl)thiazol-2-yl)ethyl)-5-(3-fluoro-4-(trifluoromethyl)phenyl)-1H-pyrrole-2-carboxamide [**53** and **54** (NBD-14188)]

Compounds **53** and **54** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **38** and acid **7**. Compounds were

purified using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1).

53: M = 646 mg. Yield = 43% (over two steps).

rt = 1.317 min. Purity = 97%. LC–MS: m/z [M+H]⁺ = 429 Da.

54: M = 495 mg. Yield = 44% (over two steps).

rt = 1.323 min. Purity = 97%. LC–MS: m/z [M+H]⁺ = 429 Da.

m.p. = 125–130°C.

¹H NMR: (DMSO, 400 MHz) δ = 1.75 (br. s, 2 H), 3.00 (dd, *J*=13.2, 7.9 Hz, 1 H), 3.13 (dd, *J*=13.3, 5.4 Hz, 1 H), 4.60 (s, 2 H), 5.19 (dd, *J*=12.8, 7.3 Hz, 1 H), 5.47 (br. s, 1 H), 6.88 (d, *J*=3.9 Hz, 1 H), 7.05 (d, *J*=3.9 Hz, 1 H), 7.54 (s, 1 H), 7.72 (t, *J*=8.1 Hz, 1 H), 7.84 (d, *J*=8.3 Hz, 1 H), 8.02 (d, *J*=12.8 Hz, 1 H), 8.65 (d, *J*=8.1 Hz, 1 H), 11.99 (br. s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 45.6, 54.6, 55.8, 110.0, 112.4 (d, *J*= 22.7 Hz), 112.9, 113.7 (dq, *J*= 32.9, 12.4 Hz), 120.6 (d, *J*= 2.9 Hz), 122.9 (q, *J*= 271.5 Hz), 127.6 (d, *J*= 3.7 Hz), 129.0, 132.1 (d, *J*= 1.5 Hz), 138.5 (d, *J*= 9.5 Hz), 139.1, 140.1, 159.4 (dq, *J*= 251.8, 2.2 Hz), 160.4, 171.9.

HRMS (ESI): m/z calcd for C₁₈H₁₇F₄N₄O₂S [M+H]⁺ 429.1003, found 429.1004.

N-(2-Amino-1-(5-(hydroxymethyl)thiazol-2-yl)ethyl)-5-(3,5-difluoro-4-(trifluoromethyl)-phenyl)-1H-pyrrole-2-carboxamide [57 (NBD-14197 and 58 (NBD-14198)]

Compounds **57** and **58** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **38** and acid **10**.

Compounds were using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1, 5:1).

57: M = 611 mg. Yield = 45% (over two steps).

rt = 1.405 min. Purity = 85%. LC–MS: m/z [M+H]⁺ = 447 Da.

58: M = 776 mg. Yield = 47% (over two steps).

rt = 1.579 min. Purity = 97%. LC–MS: m/z [M+H]⁺ = 447 Da.

m.p. = 165–170°C.

¹H NMR: (DMSO, 400 MHz) δ = 3.00 (dd, *J*=13.1, 7.9 Hz, 1 H), 3.14 (dd, *J*=13.1, 5.3 Hz, 1 H), 4.59 (s, 2 H), 5.20 (dd, *J*=12.5, 6.8 Hz, 1 H), 5.47 (br. s, 1 H), 6.99 (d, *J*=3.9 Hz, 1 H), 7.06 (d, *J*=3.9 Hz, 1 H), 7.54 (s, 1 H), 7.90 (d, *J*=12.1 Hz, 2 H), 8.70 (d, *J*=7.3 Hz, 1 H).

Three exchangeable protons are missing.

¹³C NMR: (DMSO, 100 MHz) δ = 45.3, 54.3, 55.8, 102.7 (m), 108.4 (d, J = 25.6 Hz, 2C), 111.0, 112.9, 121.9 (q, J = 272.2 Hz), 129.6, 131.2, 138.9 (t, J = 11.7 Hz), 139.0, 140.2, 159.6 (d, J = 253.2 Hz, 2C), 160.3, 171.6.

HRMS (ESI): m/z calcd for C₁₈H₁₆F₅N₄O₂S [M+H]⁺ 447.0909, found 447.0912.

N-(2-Amino-1-(5-(hydroxymethyl)thiazol-2-yl)ethyl)-5-(3,5-difluoro-4-methylphenyl)-1H-pyrrole-2-carboxamide [43 and 44 (NBD-14184)]

Compounds **43** and **44** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **38** and acid **4**. Compounds were purified using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1).

43: M = 606 mg. Yield = 51% (over two steps).

rt = 1.267 min. Purity = 100%. LC-MS: m/z [M+H]⁺ = 393 Da.

44: M = 559 mg. Yield = 42% (over two steps).

rt = 1.281 min. Purity = 100%. LC-MS: m/z [M+H]⁺ = 393 Da.

$m.p.$ = 185–190°C.

¹H NMR: (DMSO, 400 MHz) δ = 1.78 (br. s, 2 H), 2.12 (s, 3 H), 3.00 (dd, J = 13.2, 7.9 Hz, 1 H), 3.14 (dd, J = 13.6, 5.7 Hz, 1 H), 4.60 (s, 2 H), 5.20 (dd, J = 13.1, 7.6 Hz, 1 H), 5.48 (br. s, 1 H), 6.73 (d, J = 3.8 Hz, 1 H), 7.00 (d, J = 3.8 Hz, 1 H), 7.54 (s, 1 H), 7.58 (d, J = 8.5 Hz, 2 H), 8.57 (d, J = 7.9 Hz, 1 H), 11.92 (br. s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 6.8 (t, J = 3.3 Hz), 45.6, 54.5, 55.8, 107.1 (dd, J = 19.9, 8.3 Hz, 2C), 108.4, 110.4 (t, J = 21.8 Hz), 112.8, 127.8, 131.7 (t, J = 11.1 Hz), 132.8 (t, J = 2.9 Hz), 139.0, 140.1, 160.4, 161.2 (dd, J = 242.8, 10.4 Hz, 2C), 172.0.

HRMS (ESI): m/z calcd for C₁₈H₁₉F₂N₄O₂S [M+H]⁺ 393.1191, found 393.1196.

N-(2-Amino-1-(4-(hydroxymethyl)thiazol-2-yl)ethyl)-5-(4-chloro-3,5-difluorophenyl)-1H-pyrrole-2-carboxamide [39 and 40 (NBD-14169)]

Compounds **39** and **40** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **25** and acid **11**. Compounds were purified using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1).

39: M = 264 mg. Yield = 21% (over two steps).

rt = 1.399 min. Purity = 98%. LC-MS: m/z [M+H]⁺ = 413 Da.

40: M = 356 mg. Yield = 27% (over two steps).

rt = 1.337 min. Purity = 97%. LC-MS: m/z [M+H]⁺ = 413 Da.

¹H NMR (DMSO-*d*₆, 400 MHz): δ = 1.71 (br. s, 2 H), 2.99 (dd, *J*=13.2, 7.8 Hz, 1 H), 3.13 (dd, *J*=13.2, 5.4 Hz, 1 H), 4.53 (s, 2 H), 5.21 (dd, *J*=12.8, 7.7 Hz, 1 H), 5.29 (br. s, 1 H), 6.84 (d, *J*=3.8 Hz, 1 H), 7.03 (d, *J*=3.9 Hz, 1 H), 7.28 (s, 1 H), 7.86 (d, *J*=9.0 Hz, 2 H), 8.64 (d, *J*=7.7 Hz, 1 H), 12.03 (br. s, 1 H).

¹³C NMR (DMSO-*d*₆, 100 MHz): δ = 45.7, 54.4, 59.8, 105.4 (t, *J* = 21.3 Hz), 108.4 (d, *J* = 25.7 Hz, 2C), 109.5, 112.7, 114.1, 128.5, 131.8 (t, *J* = 2.8 Hz), 132.8 (t, *J* = 10.4 Hz), 157.6, 158.3 (dd, *J* = 246.2, 4.4 Hz, 2C), 160.3, 172.1.

HRMS (ESI): *m/z* calcd for C₁₇H₁₆ClF₂N₄O₂S [M+H]⁺ 413.0645, found 413.0646.

N-(2-Amino-1-(4-(hydroxymethyl)thiazol-2-yl)ethyl)-5-(4-(trifluoromethyl)phenyl)-1H-pyrrole-2-carboxamide [49 and 50 (NBD-14171)]

Compounds **49** and **50** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **25** and acid **12**. Compounds were purified using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1).

39: M = 375 mg. Yield = 34% (over two steps).

rt = 1.314 min. Purity = 100%. LC–MS: *m/z* [M+H]⁺ = 411 Da.

50: M = 666 mg. Yield = 54% (over two steps).

rt = 1.318 min. Purity = 100%. LC–MS: *m/z* [M+H]⁺ = 411 Da.

m.p. = 225–230°C.

¹H NMR (DMSO-*d*₆, 400 MHz): δ = 1.70 (br. s, 2 H), 3.00 (dd, *J*=13.2, 7.8 Hz, 1 H), 3.13 (dd, *J*=13.1, 5.3 Hz, 1 H), 4.53 (s, 2 H), 5.21 (dd, *J*=12.8, 7.7 Hz, 1 H), 5.25 – 5.31 (m, 1 H), 6.77 (d, *J*=3.8 Hz, 1 H), 7.03 (d, *J*=3.9 Hz, 1 H), 7.28 (s, 1 H), 7.70 (d, *J*=8.3 Hz, 2 H), 8.02 (d, *J*=8.2 Hz, 2 H), 8.61 (d, *J*=7.9 Hz, 1 H), 11.98 (br. s, 1 H).

¹³C NMR (DMSO-*d*₆, 100 MHz): δ = 45.7, 54.4, 59.7, 108.8, 113.0, 114.0, 124.4 (q, *J* = 271.5 Hz), 125.0 (2C), 125.6 (q, *J* = 4.0 Hz, 2C), 126.6 (q, *J* = 31.3 Hz), 128.3, 133.2, 135.6, 157.6, 160.3, 172.1.

HRMS (ESI): *m/z* calcd for C₁₈H₁₈F₃N₄O₂S [M+H]⁺ 411.1097, found 411.1096.

N-(2-Amino-1-(4-(hydroxymethyl)thiazol-2-yl)ethyl)-5-(3-fluoro-4-(trifluoromethyl)phenyl)-1H-pyrrole-2-carboxamide [55 and 56 (NBD-14190)]

Compounds **55** and **56** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **25** and acid **7**. Compounds were purified using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1).

55: M = 352 mg. Yield = 27% (over two steps).

rt = 1.336 min. Purity = 100%. LC–MS: m/z [M+H]⁺ = 429 Da.

56: M = 568 mg. Yield = 36% (over two steps).

rt = 1.336 min. Purity = 100%. LC–MS: m/z [M+H]⁺ = 429 Da.

m.p. = 180–185°C.

¹H NMR: (DMSO, 400 MHz) δ = 1.71 (br. s, 2 H), 3.00 (dd, *J*=13.2, 7.9 Hz, 1 H), 3.14 (dd, *J*=13.3, 5.4 Hz, 1 H), 4.53 (s, 2 H), 5.22 (dd, *J*=12.7, 7.3 Hz, 1 H), 5.31 (br. s, 1 H), 6.88 (d, *J*=3.9 Hz, 1 H), 7.05 (d, *J*=3.9 Hz, 1 H), 7.28 (s, 1 H), 7.72 (t, *J*=8.1 Hz, 1 H), 7.84 (d, *J*=8.4 Hz, 1 H), 8.02 (d, *J*=13.0 Hz, 1 H), 8.67 (d, *J*=7.8 Hz, 1 H), 11.76 (br. s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 45.8, 54.5, 59.8, 110.0, 112.4 (d, *J* = 22.7 Hz), 113.0, 113.6 (dq, *J* = 32.9, 12.4 Hz), 114.1, 120.6 (d, *J* = 2.2 Hz), 122.9 (q, *J* = 271.5 Hz), 127.6 (d, *J* = 3.7 Hz), 129.0, 132.1 (d, *J* = 1.5 Hz), 138.5 (d, *J* = 9.5 Hz), 157.7, 159.5 (dq, *J* = 251.0, 1.5 Hz), 160.4, 172.2.

HRMS (ESI): m/z calcd for C₁₈H₁₇F₄N₄O₂S [M+H]⁺ 429.1003, found 429.1005.

N-(2-Amino-1-(4-(hydroxymethyl)thiazol-2-yl)ethyl)-5-(3,5-difluoro-4-methylphenyl)-1H-pyrrole-2-carboxamide [45 and 46 (NBD-14186)]

Compounds **45** and **46** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **25** and acid **4**. Compounds were purified using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1).

45: M = 433 mg. Yield = 37% (over two steps).

rt = 1.246 min. Purity = 100%. LC–MS: m/z [M+H]⁺ = 393 Da.

46: M = 572 mg. Yield = 39% (over two steps).

rt = 1.326 min. Purity = 100%. LC–MS: m/z [M+H]⁺ = 393 Da.

m.p. = 180–185°C.

¹H NMR: (DMSO, 400 MHz) δ = 1.66 (br. s, 2 H), 2.12 (s, 3 H), 3.00 (dd, *J*=13.1, 7.9 Hz, 1 H), 3.13 (dd, *J*=13.3, 5.4 Hz, 1 H), 4.53 (s, 2 H), 5.21 (dd, *J*=13.0, 7.5 Hz, 1 H), 5.31 (br. s., 1 H), 6.73 (d, *J*=3.8 Hz, 1 H), 7.00 (d, *J*=3.8 Hz, 1 H), 7.28 (s, 1 H), 7.57 (d, *J*=8.6 Hz, 2 H), 8.58 (d, *J*=7.8 Hz, 1 H), 11.89 (br. s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 6.8 (t, *J* = 3.3 Hz), 45.8, 54.4, 59.8, 107.1 (dd, *J* = 19.4, 8.4 Hz, 2C), 108.4, 110.4 (t, *J* = 22.0 Hz), 112.8, 114.1, 127.8, 131.7 (t, *J* = 11.0 Hz), 132.8 (t, *J* = 2.9 Hz), 157.6, 160.4, 161.2 (dd, *J* = 243.0, 10.3 Hz, 2C), 172.3.

HRMS (ESI): m/z calcd for C₁₈H₁₉F₂N₄O₂S [M+H]⁺ 393.1191, found 393.1195.

5-(4-Chloro-3,5-difluorophenyl)-N-(1-(4-(hydroxymethyl)thiazol-2-yl)-2-(methylamino)ethyl)-1H-pyrrole-2-carboxamide [41 (NBD-14141) and 42 (NBD-14142)]

Compounds **41** and **42** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **27** and acid **11**. Compounds were purified using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1).

41: M = 656 mg. Yield = 52% (over two steps).

rt = 1.447 min. Purity = 97%. LC-MS: m/z [M+H]⁺ = 427 Da.

42: M = 852 mg. Yield = 65% (over two steps).

rt = 1.106 min. Purity = 93%. LC-MS: m/z [M+H]⁺ = 427 Da.

m.p. = 170–175°C.

¹H NMR (DMSO-*d*₆, 400 MHz) δ = 1.94 (br. s, 1 H), 2.32 (s, 3 H), 2.96 – 3.11 (m, 2 H), 4.52 (br. s, 2 H), 5.29 (br. s, 1 H), 5.42 (dd, *J*=13.4, 7.9 Hz, 1 H), 6.84 (d, *J*=3.8 Hz, 1 H), 7.01 (d, *J*=3.8 Hz, 1 H), 7.28 (s, 1 H), 7.84 (d, *J*=9.2 Hz, 2 H), 8.65 (d, *J*=7.9 Hz, 1 H), 12.02 (br. s, 1 H).

¹³C NMR (DMSO-*d*₆, 100 MHz): δ = 35.5, 50.5, 54.4, 59.7, 105.4 (t, *J*= 20.9 Hz), 108.4 (d, *J*= 25.7 Hz, 2C), 109.5, 112.7, 114.2, 128.5, 131.8 (t, *J*= 2.4 Hz), 132.8 (t, *J*= 10.4 Hz), 157.6, 158.4 (dd, *J*= 245.8, 4.0 Hz, 2C), 160.2, 172.2.

HRMS (ESI): m/z calcd for C₁₈H₁₈ClF₂N₄O₂S [M+H]⁺ 427.0802, found 427.0802.

N-(1-(4-(Hydroxymethyl)thiazol-2-yl)-2-(methylamino)ethyl)-5-(4-(trifluoromethyl)phenyl)-1H-pyrrole-2-carboxamide [51 (NBD-14161) and 52 (NBD-14160)]

Compounds **51** and **52** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **27** and acid **12**. Compounds were purified using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1).

51: M = 476 mg. Yield = 33% (over two steps).

rt = 1.322 min. Purity = 100%. LC-MS: m/z [M+H]⁺ = 425 Da.

52: M = 687 mg. Yield = 48% (over two steps).

rt = 1.292 min. Purity = 100%. LC-MS: m/z [M+H]⁺ = 425 Da.

m.p. = 190–195°C.

¹H NMR (DMSO-*d*₆, 400 MHz) δ = 1.85 (br. s, 1 H), 2.33 (s, 3 H), 3.02 (dd, *J*=12.5, 8.6 Hz, 1 H), 3.08 (dd, *J*=12.5, 5.5 Hz, 1 H), 4.54 (s, 2 H), 5.30 (br. s, 1 H), 5.40 – 5.49 (m, 1

H), 6.77 (d, $J=3.8$ Hz, 1 H), 7.03 (d, $J=3.8$ Hz, 1 H), 7.28 (s, 1 H), 7.70 (d, $J=8.3$ Hz, 2 H), 8.02 (d, $J=8.2$ Hz, 2 H), 8.64 (d, $J=8.1$ Hz, 1 H), 12.01 (br. s, 1 H).

^{13}C NMR (DMSO- d_6 , 100 MHz): δ = 35.5, 50.4, 54.6, 59.8, 108.9, 113.1, 114.1, 124.4 (q, $J=271.5$ Hz), 125.0 (2C), 125.6 (q, $J=3.7$ Hz, 2C), 126.6 (q, $J=31.5$ Hz), 128.3, 133.3, 135.6, 157.6, 160.3, 172.3.

HRMS (ESI): m/z calcd for $\text{C}_{19}\text{H}_{20}\text{F}_3\text{N}_4\text{O}_2\text{S}$ $[\text{M}+\text{H}]^+$ 425.1254, found 425.1254.

N-(2-Amino-1-(4,5-bis(hydroxymethyl)thiazol-2-yl)ethyl)-5-(3-fluoro-4-(trifluoromethyl)-phenyl)-1H-pyrrole-2-carboxamide [59 (NBD-14209 and 60 (NBD-14199))]

Compounds **59** and **60** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **29** and acid **7**. Compounds were purified two times using column chromatography on silica gel. First eluent $\text{CHCl}_3/\text{MeOH}$ saturated with NH_3 (5:1). Second eluent $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (1:1).

59: M = 264 mg. Yield = 22% (over two steps).

t_r = 1.267 min. Purity = 97%. LC-MS: m/z $[\text{M}+\text{H}]^+$ = 459 Da.

60: M = 368 mg. Yield = 34% (over two steps).

t_r = 1.262 min. Purity = 100%. LC-MS: m/z $[\text{M}+\text{H}]^+$ = 459 Da.

m.p. = 145–150 °C (decomp.).

^1H NMR: (DMSO, 400 MHz) δ = 2.99 (dd, $J=13.2$, 7.9 Hz, 1 H), 3.12 (dd, $J=13.3$, 5.4 Hz, 1 H), 4.46 (s, 2 H), 4.65 (s, 2 H), 5.08 (br. s., 1 H), 5.16 (dd, $J=12.8$, 7.2 Hz, 1 H), 5.45 (br. s., 1 H), 6.88 (d, $J=3.9$ Hz, 1 H), 7.05 (d, $J=3.9$ Hz, 1 H), 7.72 (t, $J=8.1$ Hz, 1 H), 7.84 (d, $J=8.4$ Hz, 1 H), 8.02 (d, $J=12.8$ Hz, 1 H), 8.66 (d, $J=7.8$ Hz, 1 H). Three exchangeable protons are missing.

^{13}C NMR: (DMSO, 100 MHz) δ = 45.4, 54.2, 55.1, 57.4, 110.0, 112.4 (d, $J=22.5$ Hz), 112.9, 113.7 (dq, $J=32.1$, 12.9 Hz), 120.6 (d, $J=3.2$ Hz), 122.9 (q, $J=271.5$ Hz), 127.6 (d, $J=3.2$ Hz), 129.0, 132.1, 136.2, 138.5 (d, $J=9.6$ Hz), 150.8, 159.4 (d, $J=249.0$ Hz), 160.3, 169.3.

HRMS (ESI): m/z calcd for $\text{C}_{19}\text{H}_{19}\text{F}_4\text{N}_4\text{O}_3\text{S}$ $[\text{M}+\text{H}]^+$ 459.1109, found 459.1110.

N-(2-Amino-1-(4,5-bis(hydroxymethyl)thiazol-2-yl)ethyl)-5-(4-chloro-3,5-difluorophenyl)-1H-pyrrole-2-carboxamide [61 (NBD-14208 and 62 (NBD-14207))]

Compounds **61** and **62** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **29** and acid **11**. The compound was purified two times using column chromatography on silica gel. First eluent $\text{CHCl}_3/\text{MeOH}$ saturated with NH_3 (5:1). Second eluent $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (1:1).

61: M = 346 mg. Yield = 26% (over two steps).

rt = 1.276 min. Purity = 94%. LC–MS: m/z $[M+H]^+ = 443$ Da.

62: M = 240 mg. Yield = 22% (over two steps).

rt = 1.164 min. Purity = 95%. LC–MS: m/z $[M+H]^+ = 443$ Da.

m.p. = 155–160 °C (decomp.).

¹H NMR: (DMSO, 400 MHz) δ = 2.98 (dd, J =13.2, 7.9 Hz, 1 H), 3.11 (dd, J =13.2, 5.3 Hz, 1 H), 4.45 (s, 2 H), 4.64 (s, 2 H), 5.08 (br. s., 1 H), 5.15 (dd, J =13.0, 7.3 Hz, 1 H), 5.45 (br. s., 1 H), 6.84 (d, J =3.8 Hz, 1 H), 7.03 (d, J =3.8 Hz, 1 H), 7.84 (d, J =9.2 Hz, 2 H), 8.61 (d, J =7.7 Hz, 1 H). Three exchangeable protons are missing.

¹³C NMR: (DMSO, 100 MHz) δ = 45.7, 54.5, 55.1, 57.4, 105.4 (t, J = 21.2 Hz), 108.4 (d, J = 25.6 Hz, 2C), 109.5, 112.7, 128.6, 131.8 (t, J = 2.6 Hz), 132.8 (t, J = 10.3 Hz), 136.1, 150.8, 158.4 (dd, J = 245.9, 4.4 Hz, 2C), 160.4, 169.5.

HRMS (ESI): m/z calcd for $C_{18}H_{18}ClF_2N_4O_3S$ $[M+H]^+ 443.0751$, found 443.0750.

N-(2-Amino-1-(4-(2-hydroxypropan-2-yl)thiazol-2-yl)ethyl)-5-(4-chloro-3,5-difluorophenyl)-1H-pyrrole-2-carboxamide [64 (NBD-14221 and 63 (NBD-14220)]

Compounds **64** and **63** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **31** and acid **11**. The compound was purified two times using column chromatography on silica gel. First eluent $CHCl_3$ /MeOH saturated with NH_3 (10:1). Second eluent CH_2Cl_2 /MeOH (5:1, 1:1).

64: M = 682 mg. Yield = 43% (over two steps).

rt = 1.471 min. Purity = 92%. LC–MS: m/z $[M+H]^+ = 441$ Da.

63: M = 779 mg. Yield = 41% (over two steps).

rt = 1.402 min. Purity = 95%. LC–MS: m/z $[M+H]^+ = 441$ Da.

m.p. = 180–185°C.

¹H NMR: (DMSO, 400 MHz) δ = 1.43 (d, J =3.8 Hz, 6 H), 3.01 (dd, J =13.2, 7.9 Hz, 1 H), 3.16 (dd, J =12.6, 5.8 Hz, 1 H), 5.11 (br. s., 1 H), 5.22 (dd, J =12.7, 7.4 Hz, 1 H), 6.84 (d, J =3.9 Hz, 1 H), 7.03 (d, J =3.9 Hz, 1 H), 7.21 (s, 1 H), 7.85 (d, J =9.0 Hz, 2 H), 8.69 (d, J =7.8 Hz, 1 H). Three exchangeable protons are missing.

¹³C NMR: (DMSO, 100 MHz) δ = 30.5, 30.5, 45.6, 54.4, 70.3, 105.4 (t, J =21.4 Hz), 108.4 (dd, J =24.0, 1.5 Hz, 2C), 109.5, 111.6, 112.8, 128.5, 131.9 (t, J =2.7 Hz), 132.8 (t, J =10.4 Hz), 158.4 (dd, J = 246.0, 4.3 Hz, 2C), 160.4, 164.9, 171.8.

HRMS (ESI): m/z calcd for $C_{19}H_{20}ClF_2N_4O_2S$ $[M+H]^+ 441.0958$, found 441.0968.

N-(2-Amino-1-(4-(2-methoxypropan-2-yl)thiazol-2-yl)ethyl)-5-(4-chloro-3,5-difluorophenyl)-1H-pyrrole-2-carboxamide 65 (NBD-14222)

Compound **65** was obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **32B** and acid **11**. The compound was purified two times using column chromatography on silica gel. First eluent CHCl₃/MeOH saturated with NH₃ (10:1). Second eluent CH₂Cl₂/MeOH (5:1).

65: M = 700 mg. Yield = 43% (over two steps).

rt = 1.550 min. Purity = 100%. LC–MS: m/z [M+H]⁺ = 455 Da.

m.p. = 155–160°C.

¹H NMR: (DMSO, 400 MHz) δ = 1.47 (s, 6 H), 2.99 (s, 3 H), 3.02 (dd, *J*=13.3, 8.1 Hz, 1 H), 3.17 (dd, *J*=13.3, 5.1 Hz, 1 H), 5.22 (d, *J*=12.8, 7.6 Hz, 1 H), 6.85 (d, *J*=3.9 Hz, 1 H), 7.03 (d, *J*=3.9 Hz, 1 H), 7.35 (s, 1 H), 7.87 (d, *J*=9.0 Hz, 2 H), 8.71 (d, *J*=7.7 Hz, 1 H), 12.00 (br. s, 1 H). Two exchangeable protons are missing.

¹³C NMR: (DMSO, 100 MHz) δ = 26.3 (2C), 45.6, 50.0, 54.4, 74.8, 105.4 (t, *J*=21.6 Hz), 108.4 (d, *J*=25.7 Hz, 2C), 109.5, 112.8, 114.5, 128.5, 131.9 (t, *J*=3.0 Hz), 132.8 (t, *J*=10.1 Hz), 158.3 (dd, *J*= 245.9, 4.1 Hz, 2C), 160.0, 160.4, 171.9.

HRMS (ESI): m/z calcd for C₂₀H₂₂ClF₂N₄O₂S [M+H]⁺ 455.1115, found 455.1121.

N-(2-Amino-1-(5-(hydroxymethyl)oxazol-2-yl)ethyl)-5-(4-chloro-3,5-difluorophenyl)-1H-pyrrole-2-carboxamide [67 (NBD-14175 and 66 (NBD-14174)]

Compounds **67** and **66** were obtained following the general procedure for amide coupling and then the general procedure for deprotection from amine **37** and acid **11**. Compounds were purified using column chromatography on silica gel. Eluent CHCl₃/MeOH saturated with NH₃ (10:1, 4:1).

67: M = 680 mg. Yield = 53% (over two steps).

rt = 1.237 min. Purity = 90%. LC–MS: m/z [M+H]⁺ = 397 Da.

66: M = 478 mg. Yield = 39% (over two steps).

rt = 1.289 min. Purity = 93%. LC–MS: m/z [M+H]⁺ = 397 Da.

m.p. = 115–120°C (decomp.).

¹H NMR: (DMSO, 400 MHz) δ = 1.50 – 3.50 (br. s, 2H), 2.97 (dd, *J*=13.1, 7.2 Hz, 1 H), 3.09 (dd, *J*=13.1, 6.0 Hz, 1 H), 4.42 (s, 2 H), 5.13 (q, *J*=7.0 Hz, 1 H), 5.33 (br. s, 1 H), 6.82 (d, *J*=3.9 Hz, 1 H), 6.96 (s, 1 H), 6.99 (d, *J*=3.9 Hz, 1 H), 7.83 (d, *J*=9.0 Hz, 2 H), 8.52 (d, *J*=8.3 Hz, 1 H), 11.95 (br. s, 1 H).

¹³C NMR: (DMSO, 100 MHz) δ = 44.3, 50.1, 53.4, 105.4 (t, J = 21.3 Hz), 108.4 (d, J = 24.9 Hz, 2C), 109.5, 112.7, 123.7, 128.5, 131.7 (t, J = 2.8 Hz), 132.8 (t, J = 10.0 Hz), 152.2, 158.4 (dd, J = 245.8, 4.0 Hz, 2C), 160.1, 162.8.

HRMS (ESI): m/z calcd for $C_{17}H_{16}ClF_2N_4O_3$ $[M+H]^+$ 397.0874, found 397.0875.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HIV-1	Human Immunodeficiency Virus Type 1
Env	Envelope
AIDS	acquired immunodeficiency syndrome
VSV-G	Vesicular stomatitis virus-G
ADMET	absorption, distribution, metabolism and excretion
SM	starting material
DCM	dichloromethane
DIPEA	N,N-Diisopropylethylamine
HBTU	N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
TBSCl	tert-Butyldimethylsilyl chloride
TFAA	Trifluoroacetic anhydride

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Highlights

- X-ray structure of NBD-14010-HIV-1gp120 helped to generate a hypothesis.
- The CH₂OH switch improved the antiviral activity of the entry antagonists.
- The NBD entry inhibitors showed broad-spectrum antiviral activity.
- Best inhibitors have comparable ADMET properties to BMS-626529.

Figure 1.

Structures and a sequential improvement of antiviral activity of NBD series inhibitors. The IC_{50} s were calculated from the data when tested against pseudovirus HIV-1_{HXB2}.

Figure 2. CH₂OH “positional switch” hypothesis based on the X-ray structure of NBD-14010
a) X-ray structure of NBD-14010 with HIV-1 gp120 (PDB ID:5U6E). **b)** XP docking pose of the modified structure of NBD-14010 after the positional switch of CH₂OH and removing CH₃ from the thiazole ring; **c)** Overlapped structures of NBD-14010 in 5U6E to the docked pose of modified NBD-14010 after CH₃ removal and CH₂OH switch; **d)** conversion of NBD-14010 to **39** by positional switching of CH₂OH.

Figure 3.

A. HIV-1 mediated cell-cell fusion inhibition assay. Indicator cells TZM-bl cells were cocultured with Env- and Tat-expressing HL2/3 cells in the presence of escalating concentrations of NBD compounds. Two independent experiments were performed in triplicate, and the graph is representative of one experiment; the values represent the mean $\pm$ standard deviation.

B. Infectivity of Cf2Th-CCR5 cells by CD4-dependent HIV-1_{ADA}. Cf2Th-CCR5 cells were infected with CD4-dependent HIV-1_{ADA} in the presence of NBD compounds. The Relative virus infectivity indicates the amount of infection detected in the presence of the compounds divided by the amount of infection detected in the absence of the compounds. Three independent experiments were performed in triplicate, and the graph is representative of one experiment. The toxicity of the compounds against these cells was evaluated to calculate the CC_{50} values (NBD-556: $> 60 \mu\text{M}$; NBD-14136: $37.7 \pm 1.7 \mu\text{M}$; NBD-14168: $> 51 \mu\text{M}$ and NBD-14189: $34 \pm 1 \mu\text{M}$. All the values represent the mean $\pm$ standard deviation.

Scheme 1.

Synthesis of arylpyrrole-carboxylic acids. * - yields over two steps

Scheme 2.
Synthesis of thiazoles

Scheme 3.

Synthesis of amines. * - yields over two steps

Scheme 4.

Synthesis of oxazole. * - yields over two steps

Scheme 5.
Synthesis of NBD-compounds

Table 1

Anti-HIV-1 activity (IC₅₀) and cytotoxicity (CC₅₀) of NBD compounds in single-cycle (TZM-bl cells) and multi-cycle (MT-2 cells) assays.

No.	Code (enantiomer)	X	Substituent						TZM-bl ^b			MT-2 ^b	
			R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	HIV-1 _{HXB2} IC ₅₀ (μM)	Toxicity CC ₅₀ (μM)	HIV-1 _{MB} IC ₅₀ (μM)	Toxicity CC ₅₀ (μM)	
	NBD-14106 ^a (R)	S	F	Cl	F	CH ₂ OH	H	H	0.48±0.1	20.6±0.2	0.97±0.2	17.4±0.4	
	NBD-14107 ^a (S)	S	F	Cl	F	CH ₂ OH	H	H	0.64±0.06	39.5±2.3	0.96±0.1	37±1.5	
39	NBD-14168 (S)	S	F	Cl	F	H	CH ₂ OH	H	0.28±0.02	42.1±2.6	0.21±0.001	32.6 ± 0.3	
40	NBD-14169 (R)	S	F	Cl	F	H	CH ₂ OH	H	0.5±0.01	27.7±0.3	0.49±0.02	29.6±0.5	
41	NBD-14141 (S)	S	F	Cl	F	H	CH ₂ OH	CH ₃	0.23±0.05	18±0.5	0.18±0.05	18.8±0.9	
42	NBD-14142 (R)	S	F	Cl	F	H	CH ₂ OH	CH ₃	0.25±0.04	23.8±2.8	0.4±0.07	24±1	
43	NBD-14183 (S)	S	F	CH ₃	F	CH ₂ OH	H	H	0.47±0.04	33.2±2.5	0.2±0.05	27±0.78	
44	NBD-14184 (R)	S	F	CH ₃	F	CH ₂ OH	H	H	0.20±0.05	32.9±0.6	0.2±0.04	26.8±1.8	
45	NBD-14185 (S)	S	F	CH ₃	F	H	CH ₂ OH	H	0.18±0.03	18.5±0.6	0.18±0.03	25.5±0.5	
46	NBD-14186 (R)	S	F	CH ₃	F	H	CH ₂ OH	H	0.54±0.04	20.6±0.2	0.6±0.1	15.9±0.82	
47	NBD-14135 (S)	S	H	CF ₃	H	CH ₂ OH	H	H	1.6±0.5	35.6±1	0.68±0.3	36 ± 1	
48	NBD-14136 (R)	S	H	CF ₃	H	CH ₂ OH	H	H	0.27±0.02	42.4±1	0.68±0.2	32 ± 1

X = S or O

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<div> </div> <div>X = S or O</div>													
No.	Code (enantiomer)	X	Substituent						TZM-b ^b			MT-2 ^b	
			R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	HIV-1 _{HXB2} IC ₅₀ (μM)	Toxicity CC ₅₀ (μM)	HIV-1 _{IIIB} IC ₅₀ (μM)	Toxicity CC ₅₀ (μM)	
64	NBD-14221 (S)	S	F	Cl	F	H	C(CH ₃) ₂ OH	H	0.23±0.03	28.4±0.4	0.25±0.07	16.6±0.8	
65	NBD-14222 (R)	S	F	Cl	F	H	C(CH ₃) ₂ OCH ₃	H	9.3±1.3	39.6±2.2	6.1±1.3	29.5±0.4	
66	NBD-14174 (R)	O	F	Cl	F	CH ₂ OH	H	H	0.76±0.1	30.9±1.7	0.9±0.1	44.7±1.5	
67	NBD-14175 (S)	O	F	Cl	F	CH ₂ OH	H	H	0.89±0.03	27.7±0.5	0.8±0.1	26.5±2	

^a data reported previously [27]

^b The reported IC₅₀ values represent the means ± standard deviation (SD), n=3.

Table 2
Neutralization activity of NBD compounds against a panel of HIV-1 Env Pseudoviruses

			IC ₅₀ μM ^a			
Subtype	NIH #	ENVs	NBD-14107	48	39	55
A	11887	Q259ENV.W6	0.36±0.04 ^c	0.46±0.06	0.28±0.02	0.17±0.01
	11888	QB726.70M.ENV.C4	0.51±0.03 ^c	0.4±0.02	0.23±0.02	0.098±0.002
	11891	QF495.23M.ENV.A3	0.44±0.07 ^c	0.26±0.04	0.25±0.01	0.1±0.007
	11892	QF495.23M.ENV.B2	0.68±0.06 ^c	0.15±0.02	0.099±0.003	0.14±0.003
	11894	QG984.21M.ENV.A3	0.89±0.07	0.2±0.01	0.17±0.01	0.096±0.004
	11896	QH343.21M.ENV.A10	0.72±0.01	0.32±0.01	0.21±0.02	0.093±0.001
		BG505-T332N	0.41±0.02 ^c	0.25±0.05	0.2±0.01	0.092±0.001
		KNH1144	0.53±0.09 ^c	0.38±0.06	0.26±0.04	0.11±0.001
A/D	11901	QA790.204I.ENV.A4	0.4±0.03 ^c	0.28±0.02	0.14±0.03	0.12±0.002
	11903	QA790.204I.ENV.C8	0.4±0.01 ^c	0.54±0.04	0.21±0.01	0.1±0.01
	11904	QA790.204I.ENV.E2	0.61±0.03 ^c	0.32±0.03	0.17±0.02	0.16±0.007
	11905	QG393.60M.ENV.A1	0.45±0.01	0.21±0.01	0.1±0.005	0.077±0.01
A2/D	11906	QG393.60M.ENV.B7	0.43±0.02 ^c	0.2±0.004	0.2±0.01	0.16±0.01
	11907	QG393.60M.ENV.B8	0.27±0.01 ^c	0.22±0.002	0.12±0.01	0.11±0.01
A/E	11603	CRF01_AE clone 269	0.62±0.05 ^c	0.35±0.04	0.22±0.02	0.18±0.001
		AA058	0.39±0.1 ^c	0.14±0.05	0.1±0.008	0.15±0.01
A/G	11601	CRF02_AG clone 263	0.6±0.09 ^c	0.53±0.02	0.25±0.01	0.13±0.02
	11602	CRF02_AG clone 266	0.48±0.07 ^c	0.29±0.02	0.19±0.01	0.094±0.002
	11605	CRF02_AG clone 278	0.5±0.03 ^c	0.43±0.02	0.2±0.007	0.078±0.02
B		B41	0.36±0.04 ^c	0.43±0.03	0.27±0.04	0.12±0.03
	11018	QH0692, clone 42	0.35±0.06 ^c	0.27±0.01	0.22±0.02	0.18±0.001
	11022	PVO, clone 4	0.5±0.05 ^c	0.21±0.01	0.21±0.01	0.11±0.007

Subtype	NIH #	ENVs	IC ₅₀ μ M ^a			
			NBD-14107	48	39	55
	11023	TRO, clone 11	0.68±0.1	0.24±0.01	0.19±0.001	0.093±0.001
	11024	AC10.0, clone 29	0.5±0.02 ^c	0.31±0.03	0.16±0.02	0.16±0.01
	11035	pREJO4541 clone 67	0.74±0.1 ^c	0.37±0.1	0.18±0.03	0.15±0.01
	11036	pRHPA4259 clone 7	0.57±0.08 ^c	0.24±0.03	0.15±0.02	0.15±0.01
	11037	pTHRO4156 clone 18	0.47±0.04	0.19±0.02	0.22±0.01	0.11±0.008
	11038	pCAAN5342 clone A2	0.6±0.03 ^c	0.2±0.01	0.17±0.02	0.093±0.001
	11058	SC422661.8	0.53±0.05 ^c	0.22±0.007	0.16±0.01	0.094±0.001
	11561	p1054.TC4.1499	0.61±0.09	0.32±0.02	0.14±0.005	0.091±0.001
	11563	p1058_11.B11.1550 ^b	0.34±0.01 ^c	0.22±0.03	0.097±0.003	0.1±0.002
	11571	p9014_01.TB1.4769	0.38±0.02 ^c	0.31±0.01	0.19±0.02	0.14±0.001
	11572	p9021_14.B2.4571	0.31±0.03 ^c	0.22±0.02	0.22±0.001	0.13±0.02
	11578	pWEAUd15.410.5017 ^b	0.5±0.04 ^c	0.29±0.02	0.11±0.003	0.1±0.007
	11306	Dul156, clone 12	0.62±0.2 ^c	0.19±0.02	0.094±0.003	0.093±0.001
	11307	Dul172, clone 17	0.61±0.05 ^c	0.23±0.01	0.16±0.01	0.1±0.01
	11308	Du422, clone 1	0.32±0.02 ^c	0.33±0.06	0.2±0.01	0.1±0.003
	11309	ZM197M.PB7	0.35±0.01	0.34±0.01	0.097±0.01	0.1±0.01
	11310	ZM214M.PL15	0.39±0.02	0.19±0.02	0.28±0.04	0.082±0.001
C	11312	ZM249M.PL1	0.71±0.07 ^c	0.21±0.01	0.17±0.02	0.1±0.004
	11313	ZM53M.PB12	0.64±0.01 ^c	0.19±0.002	0.15±0.02	0.17±0.01
	11314	ZM109F.PB4	0.66±0.08	0.23±0.01	0.15±0.02	0.099±0.003
	11317	CAP210.2.00.E8	0.61±0.03 ^c	0.19±0.01	0.16±0.01	0.13±0.01
	11502	HIV-16055-2, clone 3	0.35±0.01 ^c	0.29±0.04	0.15±0.01	0.12±0.04
	11504	HIV-16936-2, clone 21	0.31±0.03 ^c	0.17±0.03	0.14±0.01	0.11±0.004
	11506	HIV-25711-2, clone 4	0.52±0.06 ^c	0.22±0.01	0.22±0.01	0.1±0.003
	11507	HIV-225925-2, clone 22	0.33±0.04 ^c	0.32±0.03	0.14±0.03	0.11±0.001

			IC ₅₀ μM ^a			
Subtype	NIH #	ENVs	NBD-14107	48	39	55
D	11508	HIV-26191-2, clone 48	0.46±0.03 <i>c</i>	0.43±0.06	0.2±0.02	0.093±0.001
	11908	QB099.391M.ENV.B1	0.41±0.01	0.42±0.01	0.27±0.02	0.093±0.001
	11911	QA013.701ENV.H1	0.46±0.02 <i>c</i>	0.4±0.03	0.27±0.04	0.11±0.002
	11912	QA013.701ENV.M12	0.5±0.02 <i>c</i>	0.22±0.001	0.12±0.01	0.11±0.001
	11916	QD435.100M.ENV.B5	0.41±0.04 <i>c</i>	0.3±0.04	0.18±0.01	0.11±0.006
D/A	11917	QD435.100M.ENV.A4	0.32±0.04 <i>c</i>	0.16±0.01	0.11±0.007	0.12±0.02
	11918	QD435.100M.ENV.E1	0.5±0.05 <i>c</i>	0.36±0.02	0.35±0.02	0.14±0.008
	11526 _(Mother clone)	MF535.W0M.ENV.C1	0.36±0.03 <i>c</i>	0.2±0.01	0.17±0.004	0.063±0.002
	11517 _(Infant clone)	BF535.W6M.ENV.A1	0.55±0.1	0.49±0.07	0.14±0.03	0.084±0.004
	Mean ± SEM (μM): Overall (n=56) SI		0.5±0.02 79	0.29±0.01 146.2	0.18±0.008 233.9	0.11±0.004 199.1
Subtype A (n=8) SI		0.57±0.06 69.3	0.3±0.04 141.3	0.21±0.02 200.5	0.11±0.01 199.1	
Subtype A _{rec} (n=11) SI		0.47±0.03 84	0.32±0.04 132.5	0.17±0.02 247.6	0.12±0.01 182.5	
Subtype B (n=15) SI		0.5±0.03 79	0.27±0.02 157	0.18±0.01 233.9	0.12±0.007 182.5	
Subtype C (n=15) SI		0.49±0.04 80.6	0.26±0.02 163	0.17±0.01 247.6	0.11±0.005 199.1	
Subtype D-D/A (n=7) SI		0.49±0.04 80.6	0.3±0.05 141.3	0.19±0.03 221.6	0.11±0.01 199.1	
Control	VSV-G ^d	IC ₅₀	26±0.6 <i>c</i>	23±0.7	8.5±0.1	6±0.3
		CC ₅₀	>60	46.4±4.7	39.3±2.3	35±0.5
μM (Color code) IC ₅₀		0.2		>0.2 0.5	>0.5	

^aThe reported IC₅₀ values represent the means ± standard deviations (n = 3).

^bR5X4-tropic viruses; all the rest were CCR5-tropic viruses.

^cData previously published [24]

^dVSV-G was tested in U87-CD4-CXCR4 cells.

Table 3

Inhibitory activity against Cell-to-Cell HIV transmission by NBD compounds.

A. Chronically infected cells H9-HIV-1_{inf} and Molt-HIV-1_{ADA} were used as donor cells and TZMbl were used as acceptor cells.				
Compound	IC ₅₀ (μM) ^d			
	TZMbl-H9-HIV-1 _{inf}	TZMbl-Molt-HIV-1 _{ADA}		
NBD-14107	0.56±0.03	1.7±0.1		
48	0.41±0.07	1±0.1		
39	0.079±0.003	0.44±0.002		
55	0.097±0.006	0.3±0.02		
BMS-626529	0.025±0.005	~0.4		

B. PBMC cells acutely infected with lab-adapted and primary clinical isolates HIV-1 were used as donor cells and TZMbl were used as acceptor cells.						
Viral Strain	Subtype/coreceptor	IC ₅₀ (μM) ^d				
		NBD-14107	48	39	55	BMS-626529
HIV-1 _{93RW018}	A / R5	2.5±0.1	2.4±0.3	0.2±0.006	0.18±0.01	~0.1
HIV-1 _{93RW034}	A / R5	0.89±0.07	0.87±0.01	0.29±0.02	0.19±0.01	~0.1
HIV-1 _{LA1} ^b	B / X4	0.73±0.03	0.31±0.01	0.21±0.05	0.26±0.05	~0.1
HIV-1 _{89.6} ^b	B / R5X4	2.6±0.3	0.54±0.1	0.25±0.004	0.33±0.01	~0.1
HIV-1 _{BaL} ^b	B / R5	0.64±0.1	0.45±0.03	0.23±0.01	0.33±0.02	~0.1
HIV-1 _{92US073}	B / R5	1.2±0.05	0.66±0.05	0.22±0.01	0.16±0.001	~0.1
HIV-1 _{92US657}	B / R5	3.6±0.2	1.2±0.08	0.59±0.05	0.71±0.03	~0.1
HIV-1 _{93BR019}	BE/ X4	1.9±0.16	0.25±0.04	0.31±0.01	0.2±0.006	~0.1
HIV-1 _{CM002}	EA / X4	4.5±0.3	4.2±0.5	1.87±0.01	1.1±0.14	>6
HIV-1 _{Ru132}	G / R5	0.84±0.1	0.57±0.03	0.33±0.1	0.3±0.02	~0.1
HIV-1 _{RU570}	G / R5	1.3±0.1	0.28±0.006	0.37±0.004	0.32±0.01	~0.1
HIV-1 _{BCF01}	O / R5	0.96±0.06	0.31±0.03	0.28±0.06	0.22±0.01	>6
HIV-1 _{BCF03}	O / R5	2.2±0.06	0.71±0.05	1.6±0.08	0.18±0.01	>6
Mean ± SEM (μM): (n=13)		1.84±0.3	0.98±0.3	0.52±0.15	0.34±0.07	-

B. PBMC cells acutely infected with lab-adapted and primary clinical isolates HIV-1 were used as donor cells and TZMb-1 were used as acceptor cells.						
Viral Strain	Subtype/coreceptor	IC50 (µM) ^a				
		NBD-14107	48	39	55	BMS-626529
Toxicity in-infected PBMC : CC ₅₀ (µM)		>60	~61	~61	38.7±3.8	>10

^aThe reported IC50 values represent the means ± standard deviation (SD), n=3.

^aThe reported IC50 values represent the means ± standard deviation (n = 3).

^bLaboratory-adapted virus; all the rest are clinical isolate viruses

Table 4

Sensitivity of mutated-pseudoviruses to the NBD compounds

Substitutions	48 IC ₅₀ (μM)	39 IC ₅₀ (μM)	55 IC ₅₀ (μM)	BMS-626529 IC ₅₀ (nM)	Location
HIV-1 _{HXB2} WT	0.36±0.01	0.15±0.01	0.13±0.003	~8	-
L116P	0.54±0.05	0.15±0.005	0.14±0.001	>1000	CD4 binding site
A204D	0.52±0.05	0.87±0.2	0.65±0.05	>1000	V2
S375H	0.40±0.01	0.31±0.01	0.14±0.002	>1000	CD4 binding site
S375W	0.63±0.03	0.17±0.03	0.13±0.001	>1000	CD4 binding site
S375Y	0.44±0.01	0.3±0.01	0.19±0.02	>1000	CD4 binding site
I424F	0.55±0.01	1.3±0.5	0.88±0.1	>1000	CD4 binding site
N425K	1.5±0.1	0.37±0.03	0.18±0.03	~8	CD4 binding site
M426L	0.49±0.01	0.14±0.001	0.12±0.001	230±10	CD4 binding site
M434I	0.97±0.1	0.25±0.04	0.23±0.01	~8	CD4 binding site
F468L	0.24±0.05	0.14±0.01	0.12±0.006	~8	V5
M434I/I595F	0.3±0.02	0.16±0.01	0.12±0.001	~8	CD4 binding site/gp41
M475I	0.59±0.01	0.13±0.01	0.11±0.01	~50	CD4 binding site

The reported IC₅₀ values represent the means ± standard deviation (n = 3).

Table 5

In Vitro ADMET Profile of 55, 39, and BMS-626529

		Compound		
Assay performed	in vitro ADMET	39	55	BMS-626529
Solubility Range when prepared at 500 μ M	With 5% DMSO, pH7.4	~500	100–500	100–500
		(~206 μ g/mL)	(42.4–214 μ g/mL)	(47.3–237 μ g/mL)
	With 5% DMSO, pH7.4	100–500	100–500	<100
		(41.3–206 μ g/mL)	(42.8–214 μ g/mL)	(<47.3 μ g/mL)
Caco-2 permeability (mean P_{app} , $\times 10^{-6}$ cm/sec)	A-B (pH 7.4/7.4)	0.471	0.602	9.27
	B-A (pH 7.4/7.4)	17.0	17.7	32.0
	Efflux Ratio	36.4	30.5	3.46
	A-B (pH 7.4/7.4) with 100 μ M verapamil	0.837	0.777	13.5
	B-A (pH 7.4/7.4) with 100 μ M verapamil	11.0	10.9	22.7
	Efflux Ratio with 100 μ M verapamil	13.3	14.4	1.69
Metabolic Stability (human liver microsomes)	parent compound remaining at 120 min (% of 0 min)	90.4	88.5	71.5
	Cl_{int} (μ L/min/mg protein)	1.6	1.8	5.19
Protein binding (human plasma)	mean fraction unbound, %	0.281	0.949	13.1
Cytochrome P450 inhibition, IC_{50} (μ M)	CYP1A2 (Phenacetin)	> 25	> 25	> 25
	CYP2B6 (Bupropion)	> 25	> 25	> 25
	CYP2C8 (Paclitaxel)	> 25	> 25	> 25
	CYP2C9 (Diclofenac)	> 25	> 25	> 25
	CYP2C19 (Mephenytoin)	> 25	> 25	> 25
	CYP2D6 (Bufuralolol)	> 25	> 25	> 25
	CYP3A4 (Testosterone)	> 25	> 25	> 25
	CYP3A4 (Midazolam)	> 25	> 25	> 25
hERG channel inhibition, IC_{50} (μ M)		0.9	3.0	21.9