Novel Functionalized Amino Acids as Inhibitors of GABA Transporters with Analgesic Activity

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ABSTRACT: Neuropathic pain resistance to pharmacotherapy has encouraged researchers to develop effective therapies for its treatment. γ-Aminobutyric acid (GABA) transporters 1 and 4 (mGAT1 and mGAT4) have been increasingly recognized as promising drug targets for neuropathic pain (NP) associated with imbalances in inhibitory neurotransmission. In this context, we designed and synthesized new functionalized amino acids as inhibitors of GABA uptake and assessed their activities toward all four mouse GAT subtypes (mGAT1−4). According to the obtained results, compounds 2RS,4RS-39c (pIC50 (mGAT4) = 5.36), 50a (pIC50 (mGAT2) = 5.43), and 56a (with moderate subtype selectivity that favored mGAT4, pIC50 (mGAT4) = 5.04) were of particular interest and were therefore evaluated for their cytotoxic and hepatotoxic effects. In a set of in vivo experiments, both compounds 50a and 56a showed antinociceptive properties in three rodent models of NP, namely, chemotherapy-induced neuropathic pain models (the oxaliplatin model and the paclitaxel model) and the diabetic neuropathic pain model induced by streptozotocin; however compound 56a demonstrated predominant activity. Since impaired motor coordination is also observed in neuropathic pain conditions, we have pointed out that none of the test compounds induced motor deficits in the rotarod test.

KEYWORDS: GABA transporters, mGAT1−4 inhibitors, [3H]GABA uptake, neuropathic pain models, antiallodynic activity, antihyperalgesic activity

1. INTRODUCTION

The γ-aminobutyric acid (GABA) is a neurotransmitter known for its inhibitory modulation of neuronal networks. 1,2 Endogenous GABA is synthesized from glutamate 3,4 and controls the generation of membrane potential oscillations by acting on two types of receptors, ionotropic (GABAA) and metabotropic (GABAB) as summarized in Figure 1.5 Plasma membrane transporters of GABA (GATs) are components of one of the pathways responsible for terminating inhibitory signaling. GATs expression in different cell types is highly dynamic and can be modified depending on the activity. Reuptake achieved through GATs occurs in nerve terminals (allowing GABA to be recycled as a neurotransmitter) and/or the surrounding glial cells, whereby glial GATs are responsible for ≥20% reuptake of GABA, Figure 1.6 GABA specific transport systems represent a mechanism that regulates the efficiency of GABA transmission; thus, since 1990, the family of GAT sodium symporters has become an interesting biological target. Cloning of several GATs has led to a better understanding of the molecular properties of this solute carrier family, Figure 1.7 GATs have a confusing numbering system; hence, we present a summary of the current nomenclature based on that from the International P 記載.
Union of Basic and Clinical Pharmacology (IUPHAR). Human (and rat) GAT1 (SLC6A1), GAT2 (SLC6A13), GAT3 (SLC6A11), and BGT1 (SLC6A12) correspond to mouse mGAT1, mGAT2, mGAT3, and mGAT4, respectively. In this paper, we present the results of an in vitro test on murine GATs, and the mouse nomenclature (mGAT1–mGAT4) will be used in a later section.

mGAT1 (representing neuronal uptake) and mGAT4 (mediating transport into glial cells) are mainly localized in the central nervous system (CNS). The peripherally located mGAT2 (BGT1) is largely expressed in the liver, with lower levels observed in the kidneys and at the brain surface in the leptomeninges. mGAT3 is a second GABA transporter distributed mainly in the peripheral tissues, and since there is a lack of selective and potent mGAT3 inhibitors, the function of mGAT3 remains unclear. Due to the diversity in GAT subtype localization and function, researchers have focused on the synthesis of subtype-selective inhibitors.

Small amino acids are known to be GAT substrates; moreover, some substrate preferences toward GAT subtypes are well established. All four GATs can transport GABA (1). Additionally, β-alanine (2) is a substrate for both mGAT3 and mGAT4. 

Cutout 1. Diagram of GABA transport systems in neurons and glia (A) and stoichiometry between GABA and co-ions in GAT1 and GAT3 (B).

Figure 2. Structures and inhibitory activities (pIC50 values) of GAT substrates (1, 2, 3, 4) and GAT inhibitors (5–12) separated according to their transporter subtype selectivity: mGAT1 (tiagabine (5), DDPM-2571 (6), NO711 (8)) and mGAT4 ((S)-SNAP-5114 (9), DDPM-859 (10), DDPM-859 (11), DDPM-859 (12)).
mGAT4 with a low affinity for mGAT1 and mGAT2. A functional approach based on small molecules, such as (R)-nipecotic acid (3) or guvacine (4), has resulted in the synthesis of many subtype-selective inhibitors (Figure 2). Effective blockade of the uptake toward GAT is believed to have therapeutic value for not only epileptic seizures but also neuropathic pain (NP) and several abnormalities, including tremors, ataxia, and nervousness. mGAT1 inhibitors are the most potent compounds, and a wide range of these subtype-selective inhibitors are known (5–8, Figure 2). One example is the mGAT1 selective compound tiagabine (5), which has been approved by the FDA for adjunctive treatment of seizures. Furthermore, tiagabine (5) turned out to be highly effective in various rodent neuropathic pain models. The guvacine derivative DDPM-257 (6) is another selective mGAT1 inhibitor that is effective in mouse models of seizures, anxiety, depression, and acute and tonic pain. Moreover, mGAT4 remains a challenging target, especially since mGAT4 inhibitors seem to be suitable for antinociceptive activity. In this context, the GABA transporters were found to be interesting biological targets in the search for new treatment of NP. Nevertheless, due to the low to moderate subtype selectivity of 9–12, the development of new selective inhibitors remains an important approach for distinguishing non-mGAT1 pharmacology (Figure 2).

We previously obtained a series of GABA analogs with mGAT3/4 subtype preference with the most interesting compound 13 that could reduce tactile allodynia in neuropathic mice. In this paper, we present a continuation of our previous work with new derivatives that can be classified as

![Diagram](https://example.com/diagram.png)

**Figure 3.** Schematic summary of the structural modification approach from parent compound 13.25

**Scheme 1.** Synthesis of the 4-Hydroxypentanamide (37a–c, 39a–c, 41, 43a,b, 45) and 4-Hydroxybutanamide (47a–c) Derivatives^{44}

Reagents and conditions: (a) suitable amine (17 (A), 21 (C), 25 (D), 26 (E), or 32 (F)), TBAB, K$_2$CO$_3$, CH$_3$CN, 15 min at 0 °C and 16 h at rt; (b) argon, dry THF, reflux, 48 h.
analogs of parent compound 13. A summary of these modifications is presented in Figure 3. The first purpose of this study was to investigate how stiffening the lipophilic fragment in the second position of the N-benzyamide derivatives affects subtype preference and/or mGAT1−4 transporter inhibition compared to the more flexible analogs. Therefore, we introduced a piperidine ring to replace the flexible carbon chain (the lipophilic fragment; blue rectangle, Figure 3). To maintain an analogous structure, bisthiophene, fluorenyl, or suberenone was introduced in the 4-position of the piperidine ring (fragment 3, Figure 3). Moreover, motivated by the inhibitory activity of NO711 (8), we decided to introduce an oxime subunit into the 4-position of the 4-hydroxy- and 4-aminobutanamide derivatives. This moiety is interesting for the structure−activity relationship (SAR) discussion due to its potential ability to impact the binding mode of mGAT1 inhibitors.26 Second, on the basis of the fact that a large number of mGAT1 ligands possess a carboxylic acid fragment, we previously synthesized propanoic acid ethyl and benzyl ester derivatives for hydrolysis into the corresponding carboxylic analogs of the parent N-benamides (fragment 1, Figure 3). The last structural change was to introduce variation into the length of the main carbon chain. Therefore, the synthesized compounds are 3−5 carbon atoms in length. We exchanged the hydroxyl/amino groups for methyl or isopropyl groups to determine whether the presence of hydrogen bond donors affects GAT inhibitory potency in the present group of compounds. To explore the molecular interactions of novel obtained GABA uptake inhibitors with GABA transporters, computational docking and molecular dynamics studies have been performed. Finally, to confirm the therapeutic potential of the obtained compounds, we tested selected the most potent compounds in in vitro assays, for their antiallodynic and antihyperalgesic activities in three rodent models of NP.

2. RESULTS AND DISCUSSION

2.1. Chemistry. To synthesize the target compounds, we used secondary amines 17 (A), 20 (B), 21 (C), 25 (D), 26 (E), or 32 (F)), DIPEA, TBAB, dry DMF, reflux, 12 h.
the synthetic route presented in Scheme S1 (see Supporting Information).27−30,32−39

2.1.1. Synthesis of the 4-Hydroxypentanamide (37a−c, 39a−c, 41, 43a,b, 45), 4-Hydroxybutanamide (47a−c), 4-Aminobutanamide (50a,b), and 4-Acetamidobutanamide (51a,b) Derivatives. The designed target 4-hydroxypentanamide (37a−c, 39a−c, 41, 43a,b, 45) and 4-hydroxybutanamide (47a−c) derivatives were obtained in the two main steps. First, nucleophilic substitution between building blocks 17 (A), 21 (C), 25 (D), 26 (E), or 32 (F) and 3-bromo-5-methylidihydrofuran-2(3H)-one (34) or 3-bromomethylidihydrofuran-2(3H)-one (35) was performed to obtain compounds 36, 38, 40, 42, 44, and 46 (Scheme 1).30 Then, aminolysis was conducted following previously described synthetic procedures.23,40−43 Compounds 37a−c, 39a−c, 41, 43a,b, and 45 were isolated in pure form as a mixture of racemic diastereomers, e.g., 2RS,4RS-45 and 2RS,4SR-45, with a diastereoselectivity (ds) of approximately 7:3 (see the experimental section).

The 4-aminobutanamide derivatives (50a,b) and 4-acetamidobutanamide derivatives (51a,b) were prepared according to the synthetic route shown in Scheme 2. Compounds 50a and 50b were obtained from previously described synthetic procedures44,45 via the N-alkylation of 32 (F) with 48a,b and the hydrazinolysis of 49a,b. Acetylation of 50a and 50b gave 51a and 51b, respectively (Scheme 2).23,46

2.1.2. Synthesis of 3-Hydroxypropanamide Derivatives (54a−c, 55a−e, 56a−e, 57, 58, and 59a−c). 3-Hydroxypropanamide derivatives were obtained according to a previously reported synthetic route shown in Scheme 3.23,47−49 2-Bromo-3-hydroxypropanoic acid (52) was obtained from unprotected serine. Designed amides (53a−e) were formed after activation of acid 52 by n-propenephosphonic acid anhydride (T3P).

Alkylation of amine 17 (A), 20 (B), 21 (C), 25 (D), 26 (E), or 32 (F) by 2-bromo-3-hydroxypropanoic acid N-benzylamide (53a−e) was carried out overnight at reflux in dry dimethylformamide (DMF) with N,N-diisopropylethylamine (DIPEA) and tetra-n-butylammonium bromide (TBAB).
reduced activity toward all GABA transporter subtypes (potency comparable with parent compound hydroxybutanamide analogs (Table 1, Figure 4). Moreover, in hydroxypentanoic acid analogs 2 performed in triplicate. nd: not determined.

Table 1. Inhibitory Potencies (pIC50 ± Standard Error of the Mean (SEM)) toward mGAT1–4 Determined by the [3H]GABA Uptake Experiments and mGAT1 Binding Affinities (pKi ± SEM) from the MS Binding Assays of the Obtained Compounds

| compd        | R1 | R2 | R3 | R4   | mGAT1    | mGAT2    | mGAT3    | mGAT4    | mGAT1 |
|--------------|----|----|----|------|----------|----------|----------|----------|-------|
| 2RS,4RS-37a  | Me | A  | H  | OH   | 4.58     | 4.61     | 4.38     | 4.14     | 100 μM |
| 2RS,4SR-37a  | Me | A  | H  | OH   | 4.53 ± 0.07 | 100 μM | 4.21     | 4.56     | 100 μM |
| 2RS,4RS-37b  | Me | A  | 2-Cl| OH   | 100 μM | 52%     | 4.03     | 4.21     | 100 μM |
| 2RS,4SR-37b  | Me | A  | 2-Cl| OH   | 100 μM | 69%     | 4.14     | 100 μM | 100 μM |
| 2RS,4RS-37c  | Me | A  | 4-F | OH   | 4.75     | 4.80     | 4.94 ± 0.07 | 4.71 | 100 μM |
| 2RS,4SR-37c  | Me | A  | 4-F | OH   | 4.71 ± 0.05 | 4.23     | 4.55     | 4.8      | 100 μM |
| 2RS,4RS-39a  | Me | C  | 4-F | OH   | 100 μM | 51%     | 4.90     | 4.83     | 4.60   | 100 μM |
| 2RS,4SR-39b  | Me | C  | 4-Cl| OH   | 100 μM | 56%     | 73%      | 5.04 ± 0.10 | 100 μM |
| 2RS,4RS-39b  | Me | C  | 4-Cl| OH   | 100 μM | 51%     | 73%      | 4.99     | 100 μM |
| 2RS,4SR-39c  | Me | C  | 4-Me| OH   | 100 μM | 52%     | 5.18 ± 0.07 | 5.36 ± 0.10 | 100 μM |
| 2RS,4RS-41a  | Me | D  | H  | OH   | 100 μM | 49%     | 4.13     | 4.19     | 52%    | 100 μM |
| 2RS,4SR-41a  | Me | D  | H  | OH   | 4.07 ± 0.05 | 4.05     | 4.26     | 4.14     | 100 μM |
| 2RS,4RS-43a  | Me | E  | H  | OH   | 4.77 ± 0.05 | 4.25     | 4.67     | 4.58     | 100 μM |
| 2RS,4SR-43a  | Me | E  | H  | OH   | 4.71 ± 0.05 | 4.23     | 4.55     | 4.80     | 100 μM |
| 2RS,4SR-43b  | Me | E  | 4-F| OH   | 4.15 ± 0.05 | 100 μM | 59%     | 4.42     | 4.32   | 100 μM |
| 2RS,4SR-43b  | Me | E  | 4-F| OH   | 100 μM | 54%     | 80%      | 100 μM | 100 μM |
| 2RS,4SR-45   | Me | F  | H  | OH   | 4.36 ± 0.05 | 4.34 ± 0.07 | 4.70 ± 0.05 | 4.55 ± 0.12 | 100 μM |
| 2RS,4SR-45   | Me | F  | H  | OH   | 4.24 ± 0.05 | 4.50 ± 0.18 | 4.74 ± 0.09 | 4.40 ± 0.06 | 100 μM |
| 47a          | H  | F  | H  | OH   | 4.78 ± 0.05 | 4.44     | 4.74     | 4.83     | 100 μM |
| 47b          | H  | F  | 2-Cl| OH   | 4.50 ± 0.12 | 4.86 ± 0.10 | 5.04 ± 0.04 | 4.73 ± 0.12 | 100 μM |
| 47c          | H  | F  | 2,4-di-Cl| OH | 4.66 ± 0.05 | 4.99     | 4.96 ± 0.05 | 5.15 ± 0.04 | 100 μM |
| 50a          | H  | F  | 2-Cl| NH2  | 4.70 ± 0.06 | 5.43 ± 0.11 | 5.07 ± 0.10 | 4.69 ± 0.09 | 100 μM |
| 50b          | H  | F  | 3,4-di-Cl | NH2 | 4.91 ± 0.05 | 5.31 ± 0.05 | 5.32 ± 0.10 | 5.31 ± 0.09 | 100 μM |
| 51a          | H  | F  | 2-Cl| NH(CO)CH3 | 4.74 ± 0.05 | 4.88     | 5.03 ± 0.05 | 4.90     | 100 μM |
| 51b          | H  | F  | 3,4-di-Cl| NH(CO)CH3 | 4.93 ± 0.05 | 4.92 ± 0.06 | 5.17 ± 0.07 | 4.95     | 100 μM |

**Table 1.** Inhibitory Potencies (pIC50 ± Standard Error of the Mean (SEM)) toward mGAT1–4 Determined by the [3H]GABA Uptake Experiments and mGAT1 Binding Affinities (pKi ± SEM) from the MS Binding Assays of the Obtained Compounds.

“Data are given as the mean ± SEM of three independent experiments that were performed in triplicate. The results presented as a percent represent [3H]GABA uptake or NO711 binding in the presence of 100 μM inhibitor. Data without the SEM imply that only one experiment was performed in triplicate. nd: not determined.

(C) moiety ((2RS,4RS/2RS,4SR)-39a–c) and diphenylmethaneone O-(2-(methylamino)ethyl) oxime (F) moiety ((2RS,4RS/2RS,4SR)-45) in general showed inhibitory potency comparable with parent compound 13 except for compounds with a more rigid moiety such as 4-(bis(3-methylthiophen-2-yl)methylene)piperidine (A) moiety ((2RS,4RS/2RS,4SR)-37a–c), 4-(suberone)piperidine (E) moiety ((2RS,4RS/2RS,4SR)-43a,b), or 4-(fluoreno)piperidine (D) moiety ((2RS,4RS/2RS,4SR)-41), which displayed reduced activity toward all GABA transporter subtypes (Figure 4, Table 1). On the other hand, when we compared 4-hydroxybutanamide derivatives with diphenylethanone O-(2-(methylamino)ethyl) oxime (F) moiety 47a–c with 4-hydroxypentanoic acid analogs 2RS,4RS-45 and 2RS,4SR-45, slightly higher inhibitory activity was observed for the 4-hydroxybutanamide analogs (Table 1, Figure 4). Moreover, in the group of oximes containing benzamides ((2RS,4RS/2RS,4SR)-45, 47a–c, 50a,b, and 51a,b), the 4-amino-butanamide derivatives (50a,b) also displayed slightly improved inhibitory activity. Hence, among the oxime derivatives, compound 50a with a chlorine atom in the 2-position of the benzyl moiety exhibited the highest inhibitory potency toward mGAT2 among all compounds obtained (50a, pIC50(mGAT2) = 5.43). Acylation of the amino functionality at the 4-position of butanoic acid (51a,b) did not change the inhibitory potency profile against mGAT1–4 (Table 1, Figure 4). An interesting effect was observed for derivatives 39a–c with a suberone-N-methylpropan-1-amine (C) moiety. In this group of compounds, we identified two dual mGAT3/4 subtype selective inhibitors (2RS,4RS-39b and 2RS,4SR-39b) with a similar GAT preference to that of (S)-SNAP-5114 (9) but with reduced potency. On the other hand, compound 2RS,4RS-39c, with a methyl group in the para position of the benzyl moiety, was found to be the most potent mGAT4 inhibitor (pIC50(mGAT4) = 5.36) with favorable GAT subtype selectivity.

The exchange of a hydroxyl group for a methyl group is represented in many of the 3-hydroxypropanamide derivatives (54a–c, 55a–e, 56a–e, 57, 58, and 59a–c) and propanoic acid and butanoic acid derivatives (57, 64a,b, 65, 66a,b, 67, 68a,b, 69, 70, and 71).
IC₅₀ value is lower than 4.00.

5114 (4-aminobutanamide (64a), mGAT1-selective butanoic acid derivative (pIC₅₀(mGAT1) = 5.15) has a rigid bisthiophene moiety. (a) The model of the human and mouse in the same place and with comparable transporter activity when the compounds could bind to the transporters. We assumed that the compounds could bind to the activity of the compounds was tested on mouse used, as they are the targets for the new inhibitors. Although models of human GAT-1, BGT-1, GAT-2, and GAT-3 were generally observed for the α carbon 5 isomers. Modeling of the GABA transporters and the α positions. Surprisingly, the small structural change in 64a, with a hydrogen substituted for the chlorine atom in the N-benzylamide moiety (64b), results in a loss of activity. Unfortunately, we failed to observe an increase in inhibitory activity when the N-benzylamide moiety was exchanged for a carboxylic acid group (74 and 75), ethyl ester (71 and 73), or benzyl ester (72) (Table 3).

2.3. Molecular Modeling. To determine the binding mode of the tested compounds with GATs, molecular modeling calculations were performed. For this purpose, models of human GAT-1, BGT-1, GAT-2, and GAT-3 were used, as they are the targets for the new inhibitors. Although the activity of the compounds was tested on mouse transporters, we assumed that the compounds could bind to human and mouse in the same place and with comparable affinity due to only slight differences in the amino acid sequences, mainly concerning the N- and C-terminus.

Homology modeling of the GABA transporters and the differences between the structures of the particular types of these proteins were described in more detail in our previous work.

Molecular docking studies indicated that compounds generally bind in a similar manner in all types of transporters (Figure 6). The compounds are located along the vestibule of the transporters, which is consistent with our previous results obtained for similar 4-amino- and 4-hydroxybutanamide derivatives. Molecular dynamics simulations were performed on representatives of the most active compounds toward particular types of transporters to confirm the stability of the presented binding modes and created interactions (Figure 7, Figure S1). For compounds with an undefined absolute configuration, all possible stereoisomers were investigated. For the tested compounds, more consistent arrangements and beneficial interactions among all types of transporters were generally observed for the α carbon 5 isomers.

In the case of GAT-1, the bisthiophene fragment in the most active compound 64a creates hydrophobic interactions and CH−π stacking with TYR452 and PHE294 near the entrance to the transporter (Figure 6, panel A). The amino group of 64a is located between the aromatic ring of PHE294 and the carboxyl moiety of ASP451, i.e., residues that are part of the extracellular gate. This arrangement allows for the formation of a stable ionic bond with ASP451 and cation−π interaction with PHE294. During molecular dynamics simulation, as a result of the bending of the nonhelical fragment of domain 10, the side chain of SER454 approached the protonated amino group of the compound. This enabled the creation of a stable hydrogen bond between these groups (Figure 7, panel A). The ethyl substituent in the α position reaches TRP68, creating hydrophilic interactions. The amide carbonyl group forms a hydrogen bond with the side chain of SER456. During the dynamic calculations, a hydrogen bond with the hydroxyl group of SER454 was also observed. The benzyl fragment reaches into the S1 site, creating hydrophobic interactions mainly with LEU300, LEU460, LEU136, and PHE294. This arrangement appears to be beneficial considering that the S1 site in GAT-1 is the most hydrophobic among all types of transporters.

The diphenylmethylidene fragment of compound 50a, the most active toward BGT-1, locates itself in this transporter close to the EL6 loop compared to the poses observed in GAT-1. Compound 50a participates in hydrophobic inter-
actions mainly with TYR520, TRP540, TYR454, ILE459, and TYR453 as well as π−π stacking with TYR453 (Figure 6, panel B). The protonated primary amine is engaged in a salt bridge with ASP452. The amide group is located close to SER457, which enables the creation of a hydrogen bond during molecular dynamics simulation (Figure 7, panel B). During the simulation, a change in the conformation of the ARG61 side chain was also observed. This provided an additional hydrogen bond with the aforementioned amide group. The 2-chlorobenzyl fragment is located, contrary to that observed in the GAT-1 transporter, above the extracellular gate in the S2 site, creating hydrophobic interactions mainly with TYR133, TYR132, and TRP60. This arrangement is beneficial because the S1 site in BGT-1 is more polar than in other types of transporters. Additionally, a halogen bond between the chlorine atom of 50a and the carboxyl group of ASP452 was observed. In the case of another relatively highly active compound 50b, the diphenylmethylidene and 4-amino-butanamide fragment retain the interactions described above, whereas the 3,4-dichlorobenzyl fragment is located at the level of the extracellular gate, creating hydrophobic and CH−π interactions with TYR133.

In GAT-2, the most active compound 50b is placed similarly as in the BGT-1 transporter. The diphenylmethylidene fragment forms hydrophobic interactions mainly with TYR448, TYR515, and MET454. The protonated amino group of this compound creates a stable salt bridge with ASP447 (Figure 6, panel C). The amide moiety is located near the nonhelical fragment of TM10. During the molecular dynamics simulation, the fragment containing this moiety rotates which enables creation of a hydrogen bond between the carbonyl oxygen of the amide group and the hydroxyl group of SER452. In contrast to the position in the BGT-1 transporter, the 3,4-dichlorobenzyl fragment reaches the inside of the S1 site in GAT-2. It forms hydrophobic interactions mainly with LEU294, LEU456, and PHE288.

In the case of GAT-3, the diaromatic fragments of the most active compounds 50b and 2RS,4RS-39c are in a similar position compared to that observed in the GAT-2 transporter. However, due to the presence of SER468 and PHE531, which are replaced by tyrosine and serine residues, respectively, in the other transporters, the diphenylmethylidene and dibenzocycloheptadiene fragments are bound slightly higher within the vestibule. These moieties engage in hydrophobic interactions mainly with the above-mentioned PHE531, as well as with

Table 2. Inhibitory Potencies (pIC_{50} ± SEM) toward mGAT1–4 Determined from the [3H]GABA Uptake Experiments and mGAT1 Binding Affinities (pK_{i} ± SEM) from the MS Binding Assays of the Obtained Compounds

| compd R^1 R^2 | pIC_{50} ± SEM | pK_{i} ± SEM |
|---------------|---------------|--------------|
| 54a A H | 4.82 4.78 4.79 4.35 | 100 μM: 86% |
| 54b A 2-Cl | 4.77 4.86 4.79 4.54 | 100 μM: 96% |
| 55a B H | 4.76 4.58 4.80 5.08 ± 0.04 | 100 μM: 93% |
| 55b B 2-Cl | 4.59 ± 0.04 4.80 ± 0.18 4.97 ± 0.08 5.02 ± 0.10 | 100 μM: 65% |
| 55c B 4-Cl | 4.54 ± 0.08 4.22 ± 0.09 4.82 ± 0.06 4.78 ± 0.07 | 100 μM: 49% |
| 55d B 4-F | 4.58 ± 0.05 4.69 ± 0.11 4.98 ± 0.09 4.77 ± 0.01 | 100 μM: 90% |
| 55e B 4-Me | 4.57 ± 0.05 4.67 ± 0.10 4.80 ± 0.07 4.88 ± 0.08 | 4.53 ± 0.05 |
| 56a C H | 4.67 4.56 4.64 5.04 ± 0.04 | 100 μM: 68% |
| 56b C 2-Cl | 100 μM: 50%: 4.59 ± 0.08 4.98 ± 0.02 4.56 ± 0.05 | 100 μM: 86% |
| 56c C 4-Cl | 4.21 4.53 4.84 4.99 ± 0.13 n=4 | 100 μM: 87% |
| 56d C 4-F | 4.75 4.67 4.89 ± 0.04 4.91 ± 0.09 | 100 μM: 96% |
| 56e C 4-Me | 4.73 4.54 4.69 4.95 ± 0.09 | 100 μM: 4.16 |
| 57 D H | 4.61 4.75 4.16 4.44 | 100 μM: 84% |
| 58 E H | 4.45 4.65 4.68 4.63 | 100 μM: 83% |
| 59a F H | 4.64 4.64 4.23 4.57 | 100 μM: 80% |
| 59b F 2-Cl | 4.35 4.89 4.8 4.89 | 100 μM: 92% |
| 59c F 4-F | 4.58 4.77 4.71 4.78 | 100 μM: 84% |

Table 3. Inhibitory Potencies (pIC_{50} ± SEM) toward mGAT1–4 Determined from the [3H]GABA Uptake Experiments and mGAT1 Binding Affinities (pK_{i} ± SEM) from the MS Binding Assays of the Obtained Compounds

| compd R^1 R^2 n | pIC_{50} ± SEM | pK_{i} ± SEM |
|-----------------|---------------|--------------|
| 64a H A 1 | 5.15 ± 0.08 100 μM: 108% 100 μM: 69% 100 μM: 77% | 100 μM: 81% |
| 64b 2-Cl A 1 | 100 μM: 66% 100 μM: 101% 100 μM: 61% 100 μM: 64% | 100 μM: 108% |
| 65 H F 0 | 4.23 4.58 4.86 4.58 | 100 μM: 104% |
| 66a H F 1 | 100 μM: 50% 100 μM: 55% | 4.78 4.86 | 100 μM: 104% |
| 66b 2-Cl F 1 | 100 μM: 59% 100 μM: 59% 4.34 4.92 | 100 μM: 83% |
| 67 H G 0 | 4.61 4.88 4.87 4.92 | 100 μM: 83% |
| 68 H G 1 | 4.57 4.4 4.51 4.89 | 100 μM: 81% |
TYR535 and TYR469. The protonated amino group forms an ionic bond (compound 2RS,4RS-39c) or a salt bridge (compound 50b) with ASP467, similar to the previously described compounds (Figure 6, panel D). During the molecular dynamics simulation performed for compound 2RS,4RS-39c, this protonated amino group slightly moves away from ASP467 while simultaneously approaching the aromatic ring of PHE308, which enables cation–π interaction while maintaining an ionic bond with ASP467 (Figure 7, panel D). At the same time the hydroxyl moiety creates a stable hydrogen bond with ASP467. The amide groups of both described compounds are also located close to ASP467 being involved in the hydrogen bond with this residue. However, over the course of the dynamics simulation for 2RS,4RS-39c it was observed that the amide moiety can move closer to the side chain of TYR146, creating a hydrogen bond. The 4-methylbenzyl and 3,4-dichlorobenzyl fragments are located at the level of the lower part of the extracellular gate, creating hydrophobic and CH–π interactions with TYR147. Compound 50b additionally forms a halogen bond with the amide moiety of GLY71.

2.4. Hepatotoxicity and Cytotoxicity. In this study we investigated three representative compounds, 2RS,4RS-39c, 50a, and 56a, for in vitro studies to verify their safety in HepG2 and HEK-293 cells. Among all obtained compounds, compound 2RS,4RS-39c, a 4-hydroxypentanamide derivative, and compound 50a, a 4-aminobutanamide derivative, were selected for further studies because they provided the highest inhibitory potencies.

Table 3. Inhibitory Potencies (pIC_{50} ± SEM) toward mGAT1–4 Determined from the [3H]GABA Uptake Experiments and mGAT1 Binding Affinities (pK_{i} ± SEM) from the MS Binding Assays of the Obtained Compounds

| compd | R^1  | R^2  | pIC_{50} ± SEM | pK_{i} ± SEM |
|-------|------|------|----------------|-------------|
| 71    | –CH_{2}CH_{3} | B    | 100 μM: 52% | 4.66 | 100 μM: 97% | 5.24 ± 0.05 |
| 72    | –CH_{2}Ph      | B    | 100 μM: 55% | 4.62 | 100 μM: 77% | 8.79 ± 0.02 |
| 73    | –CH_{2}CH_{3}   | G    | 100 μM: 55% | 4.62 | 100 μM: 77% | 8.79 ± 0.02 |
| 74    | H              | G    | 100 μM: 55% | 4.62 | 100 μM: 77% | 8.79 ± 0.02 |
| 75    | H              | B    | 100 μM: 71% | 4.62 | 100 μM: 77% | 8.79 ± 0.02 |
| tiagabine (5)^{19} | | | 6.88 ± 0.12 | 4.62 | 100 μM: 77% | 8.79 ± 0.02 |
| (S)-SNAP-5114 (3)^{19} | | | 4.07 ± 0.07 | 4.62 | 100 μM: 77% | 8.79 ± 0.02 |
| DPPM-859 (4)^{22} | | | 4.19 ± 0.07 | 4.62 | 100 μM: 77% | 8.79 ± 0.02 |
| DPPM-2571 (5)^{20} | | | 8.27 ± 0.03 | 4.62 | 100 μM: 77% | 8.79 ± 0.02 |
| 13^{3} | | | 8.26 ± 0.03 | 4.62 | 100 μM: 77% | 8.79 ± 0.02 |

Data are given as the mean ± SEM of three independent experiments that were performed in triplicate. The results presented as a percent represent [3H]GABA uptake or NO711 binding in the presence of 100 μM inhibitor. Data without the SEM imply that only one experiment was performed in triplicate. nd: not determined.
inhibitory activity toward mGAT4 (pIC$_{50}$ = 5.36 ± 0.10) or mGAT2 (pIC$_{50}$ = 5.43 ± 0.11), respectively. Compound 56a, a 3-hydroxypropanamide derivative, was selected as one of two compounds with moderate subtype selectivity for mGAT4.

To investigate the safety of compounds 2RS,4RS-39c, 50a, and 56a, a HepG2 hepatoma cell-based hepatotoxicity assay was used. Compounds were tested at six concentrations (0.1–100 μM). The results showed that at lower compound concentrations (0.1 and 1 μM), none of the tested compounds caused a statistically significant decrease in HepG2 cell viability and thus were not hepatotoxic in comparison to doxorubicin (DX) at 1 μM (Figure 8, panel A). However, only compound 56a did not induce significant hepatotoxicity after 72 h of incubation at concentrations up to 25 μM. A statistically significant ($p < 0.0001$) decrease in HepG2 cell viability was observed for 56a only at the highest concentrations of 50 and 100 μM (Figure 8, panel A). Compound 2RS,4RS-39c was slightly more toxic than 56a, as it showed a statistically significant ($p < 0.0001$) decrease in HepG2 cell viability at 25 μM, whereas 56a showed 100% viability compared to the control at this concentration (1% DMSO in culture media). Compound 50a significantly eradicated cell viability at concentrations between 10 and 100 μM ($p < 0.0001$). Nevertheless, these results are in accordance with the hepatotoxicity examination of thioridazine, an antipsychotic agent that is still in use (Figure 8, panel C).$^{52}$

Subsequently, a similar study was performed with the HEK-293 cell line. Overall, compounds 2RS,4RS-39c and 56a showed stronger toxic effects than in the HepG2 assay, where a significant ($***p < 0.0001$) decrease in HEK-293 cell viability was observed at the concentration of 25 μM. On

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**Figure 6.** Binding modes of compound (S)-64a in GAT-1 (A); compound (S)-50a in BGT-1 (B); compound (S)-50b in GAT-2 (C); compound (2S,4S)-39c in GAT-3 (D). CH–π and π–π interactions are marked with blue dashes, ionic interactions with pink dashes, cation–π interactions with green dashes, and hydrogen bonds with yellow dashes.
the other hand, compound 50a showed a comparable safety profile in HEK-293 and HepG2 cells.

Considering the observed antiproliferative effects of compounds 2RS,4RS-39c and 56a at 25 μM and at 1 μM for compound 50a, it can be generalized that these effects were still lower than those for DX (at 1 μM) or comparable with those of thioridazine (over the concentration range of 10−100 μM). In this respect and based on the very promising biological results, compounds 2RS,4RS-39c, 50a, and 56a were selected for further investigation to elevate their antinociceptive activity in mouse models of NP. However, taking into account the obtained range of toxicity of the tested compounds, we assumed that further drug-like property optimization is required to obtain an acceptable safety profile, which will be the next stage of our research.

2.5. In Vivo Pharmacological Evaluation (Mouse Models of Neuropathic Pain). In this part of the present research, we assessed if the compounds 2RS,4RS-39c, 50a, and 56a display analgesic (antiallodynic and antihyperalgesic) properties in NP conditions. For this purpose, we used three mouse models of NP, namely, chemotheraphy-induced NP models (i.e., the oxaliplatin model and the paclitaxel model) and the diabetic NP model induced by streptozotocin (STZ). We assessed the effect of the test compounds on tactile allodynia and thermal (heat or cold) hyperalgesia in the von Frey, hot plate, or cold plate tests, respectively. Since oxaliplatin is responsible for inducing cold hypersensitivity, in both humans and experimental animals, the cold plate test was used to assess the effect of the test compounds on the thermal pain threshold in oxaliplatin-treated mice. In the two other NP models the hot plate test was applied to measure heat pain threshold in paclitaxel- and STZ-treated mice. Since impaired motor coordination is also observed in NP conditions, we additionally tested the influence of compounds 2RS,4RS-39c, 50a, and 56a on motor coordination in the rotarod test.

2.5.1. Oxaliplatin-Induced Peripheral Neuropathy: Influence on Tactile Allodynia (von Frey Test). In the early phase of oxaliplatin-induced neuropathy, an overall effect of treatment on the mechanical nociceptive threshold was observed (2RS,4RS-39c, F[1,904, 23.80] = 180.6, p < 0.0001; 50a, F[3,037, 24.30] = 136.7, p < 0.0001; 56a, F[2,371, 21.34] = 83.50, p < 0.0001). In this early phase of neuropathy, the administration of oxaliplatin significantly lowered the pain threshold for mechanical stimulation (p < 0.0001 vs vehicle-treated nonneuropathic mice) (Figure 9). Compound 2RS,4RS-39c was not effective in this phase of oxaliplatin-induced neuropathy (Figure 9, panel A). Compound 50a at both doses significantly elevated the pain threshold for mechanical stimulation (p < 0.01 vs predrug paw withdrawal...
Additionally, both doses of compound 56a reduced tactile allodynia in the acute phase of oxaliplatin-induced neuropathy (p < 0.05 vs predrug paw withdrawal threshold) (Figure 9, panel C).

In the late phase of oxaliplatin-induced neuropathy, an overall effect of treatment on the mechanical nociceptive threshold was observed (2RS,4RS-39c, F[1.705, 13.64] = 282.0, p < 0.0001; 50a, F[2.354,18.83] = 83.09, p < 0.0001; 56a, F[2.651, 23.86] = 108.3, p < 0.0001). In this phase, only compound 56a at a dose of 10 mg/kg showed antiallodynic properties (significant at p < 0.05 vs predrug paw withdrawal threshold) (Figure 9, panel C).

2.5.2. Oxaliplatin-Induced Peripheral Neuropathy: Influence on Cold Hyperalgesia (Cold Plate Test). In the early phase of oxaliplatin-induced neuropathy, an overall effect of treatment on the thermal (cold) nociceptive threshold was observed (2RS,4RS-39c, F[1.393, 16.02] = 30.05, p < 0.0001; 50a, F[2.634,21.07] = 16.57, p < 0.0001; 56a, F[2.701, 24.31] = 27.96, p < 0.0001). In this phase, the administration of oxaliplatin significantly lowered the pain threshold for cold stimulation (p < 0.001 vs vehicle-treated nonneuropathic mice) (Figure 10). Compounds 2RS,4RS-39c and 50a were not effective in this phase of oxaliplatin-induced neuropathy (Figure 10, panels A and B). Compound 56a at a dose of 30 mg/kg reduced cold hyperalgesia in the acute phase of oxaliplatin-induced neuropathy (p < 0.05 vs predrug paw withdrawal threshold) (Figure 10, panel C).

In the late phase of oxaliplatin-induced neuropathy, an overall effect of treatment was observed (2RS,4RS-39c, F[1.171, 14.64] = 23.22, p < 0.001; 50a, F[3.125, 24.38] = 9.974, p < 0.001; 56a, F[2.508, 22.57] = 16.37, p < 0.0001). In vehicle-treated mice (VEH), pain sensitivity threshold measurements were taken in the same manner and at the same time points as in the oxaliplatin-treated groups, but vehicle-treated mice were not treated with oxaliplatin; n = 8−10.
Figure 10. continued

Statistical analysis: one-way analysis of variance followed by Tukey’s post hoc comparison. Significance vs latency of control, nonneuropathic mice: ## $p < 0.001$, ### $p < 0.0001$. Significance vs predrug (after oxaliplatin) latency to pain reaction: * $p < 0.05$. In the vehicle-treated mice (VEH), measurements of the pain sensitivity threshold were taken in the same manner and at the same time points as in the oxaliplatin-treated groups, but vehicle-treated mice were not treated with oxaliplatin; $n = 8–10$.

this phase, none of the tested compounds showed antihyperalgesic properties (Figure 10).

On the basis of the results obtained in the oxaliplatin-induced neuropathic pain model, i.e., due to lack of activity of 2RS,4RS-39c, for further pain tests and NP models, only the compounds 50a and 56a were selected.

2.5.3. Paclitaxel-Induced Peripheral Neuropathy: Influence on Tactile Allodynia (von Frey Test). In the paclitaxel-induced NP model, the effect of 50a and 56a on mechanical nociceptive threshold was assessed at two time points, i.e., on the day of paclitaxel administration (4 h after paclitaxel administration) and 7 days later. On the day of paclitaxel administration, in the von Frey test an overall effect of treatment was observed for 50a ($F[4, 35] = 4.854, p < 0.01$) and 56a ($F[4, 37] = 5.655, p < 0.01$). The post hoc analysis revealed that compared to vehicle-treated nonneuropathic mice, paclitaxel significantly lowered mechanical nociceptive threshold in mice ($p < 0.05$). The comparison between predrug and postdrug paw withdrawal thresholds in each experimental group revealed that on the day of paclitaxel administration neither 50a nor 56a at doses 10 and 30 mg/kg was able to elevate the mechanical nociceptive threshold in paclitaxel-treated mice (Figure 11, panel A).

Seven days after paclitaxel administration, an overall effect of treatment was noted for 50a and 56a ($F[4, 35] = 13.76, p < 0.0001$, and $F[4, 37] = 18.69, p < 0.0001$, respectively). On this day of experiment vehicle-treated nonneuropathic mice had still significantly elevated mechanical nociceptive threshold as compared to paclitaxel treated mice ($p < 0.01$ vs predrug, i.e., before compound 50a or 56a administration, values of paw withdrawal thresholds). Of note, the comparison of predrug and postdrug paw withdrawal thresholds in 50a-treated neuropathic mice and in 56a-treated neuropathic mice showed that on day 7 after paclitaxel injection both compounds 50a and 56a at the dose of 30 mg/kg elevated mechanical nociceptive threshold ($p < 0.05$). The lower dose of 50a or 56a was not effective in the von Frey test (Figure 11, panel B).

2.5.4. Paclitaxel-Induced Peripheral Neuropathy: Influence on Heat Nociceptive Threshold (Hot Plate Test). Both compounds 50a and 56a were also assessed for their ability to affect thermal (heat) nociceptive threshold in paclitaxel-treated mice. As shown in Figure 12 (panel A), on the day of paclitaxel administration a significantly prolonged latency to pain reaction and an increased heat nociceptive threshold were noted in all groups treated with this taxane derivative ($p < 0.05$ vs vehicle-treated nonneuropathic mice). This effect indicated that paclitaxel induced hypoalgesia in mice, which was noted on the day of paclitaxel administration but not 7 days later (Figure 12, panel A vs panel B).

On the day of paclitaxel administration, in the hot plate test, one-way ANOVA showed an overall effect of treatment for 50a ($F[4, 39] = 6.075, p < 0.001$) and 56a ($F[4, 39] = 14.27, p <
The post hoc analysis demonstrated that in paclitaxel-treated mice the compound 50a reduced latency to pain reaction at the dose of 30 mg/kg ($p < 0.05$ vs predrug latency). In vehicle-treated mice (VEH), pain sensitivity threshold measurements were taken in the same manner and at the same time points as in the paclitaxel-treated groups, but vehicle-treated mice were not treated with paclitaxel; $n = 8–10$.

Figure 11. Effects of intraperitoneally administered 50a and 56a on the mechanical nociceptive threshold in a mouse paclitaxel-induced NP model measured using the von Frey test on the day of paclitaxel injection (A) and 7 days after single-dose paclitaxel injection (B). The results are shown as the mean ($\pm$SEM) force applied to elicit paw withdrawal. Statistical analysis: one-way analysis of variance followed by Tukey’s post hoc comparison. Significance vs paw withdrawal threshold of control, nonneuropathic mice: $^*p < 0.05$, $^{##}p < 0.01$. Significance vs predrug (after paclitaxel) paw withdrawal threshold: $^*p < 0.05$. In vehicle-treated mice (VEH), pain sensitivity threshold measurements were taken in the same manner and at the same time points as in the paclitaxel-treated groups, but vehicle-treated mice were not treated with paclitaxel; $n = 8–10$.

Figure 12. Effects of intraperitoneally administered 50a and 56a on the thermal (heat) pain threshold in a mouse paclitaxel-induced NP model measured using the hot plate test on the day of paclitaxel injection (A) and 7 days after paclitaxel injection (B). The results are shown as the mean ($\pm$SEM) latency to pain reaction. Statistical analysis: one-way analysis of variance followed by Tukey’s post hoc comparison. Significance vs latency of control, nonneuropathic mice: $^*p < 0.05$. Significance vs predrug (after paclitaxel) latency to pain reaction: $^*p < 0.05$, $^{**}p < 0.01$, $^*p < 0.001$. In the vehicle-treated mice (VEH), measurements of the pain sensitivity threshold were taken in the same manner and at the same time points as in the paclitaxel-treated groups, but these vehicle-treated mice were not treated with paclitaxel; $n = 8–10$.

Figure 13. Effects of intraperitoneally administered 50a and 56a on the mechanical nociceptive threshold measured using the von Frey test (A) and effects of 50a and 56a on the thermal nociceptive threshold measured using the hot plate test (B) in a mouse STZ-induced NP model. The results are shown as the mean ($\pm$SEM) force applied to elicit paw withdrawal or the mean ($\pm$SEM) latency to pain reaction. Statistical analysis: one-way analysis of variance followed by Tukey’s post hoc comparison. Significance vs paw withdrawal threshold of control, normoglycemic (nonneuropathic) mice: $^{###}p < 0.0001$. Significance vs predrug (after STZ) paw withdrawal threshold: $^{***}p < 0.001$, $^{****}p < 0.0001$. In vehicle-treated mice (VEH), pain sensitivity threshold measurements were taken in the same manner as in the STZ-treated groups, but vehicle-treated mice were not treated with STZ; $n = 8–10$. 

The post hoc analysis demonstrated that in paclitaxel-treated mice the compound 50a reduced latency to pain reaction at the dose of 30 mg/kg ($p < 0.05$ vs predrug latency to pain reaction; Figure 12, panel A) and 56a reduced latency to pain reaction at doses 10 mg/kg ($p < 0.001$ vs predrug latency).
latency to pain reaction) and 30 mg/kg (p < 0.01 vs predrug latency to pain reaction; Figure 12, panel A).

Seven days after paclitaxel administration, one-way ANOVA did not show an overall effect of treatment for 50a (F[4,35] = 1.407, p > 0.05) in the hot plate test. In contrast to this, in this assay, an overall effect of treatment was noted for 56a (F[4, 37] = 2.765, p < 0.05). At this time point of testing, Tukey’s post hoc analysis did not reveal the effect of paclitaxel and compounds 50a and 56a on the thermal nociceptive threshold in the hot plate test (Figure 12, panel B).

2.5.5. Diabetic, STZ-Induced Peripheral Neuropathy: Influence on Tactile Allodynia (von Frey Test). In the mouse model of painful diabetic neuropathy induced by STZ, an overall effect of treatment was demonstrated for both 50a and 56a in the von Frey test (F[4, 42] = 33.02, p < 0.0001, and F[4, 40] = 26.59, p < 0.0001, respectively). As shown in Figure 13 (panel A), STZ lowered mechanical nociceptive threshold in mice (p < 0.0001 vs normoglycemic control). In the von Frey test, compounds 50a and 56a were effective only at the dose of 30 mg/kg (50a, p < 0.001; 56a, p < 0.0001 vs predrug paw withdrawal in the individual group).

2.5.6. Diabetic, STZ-Induced Peripheral Neuropathy: Influence on Heat Hyperalgesia (Hot Plate Test). In the mouse model of painful diabetic neuropathy induced by STZ, an overall effect of treatment on the heat nociceptive threshold was not demonstrated for both 50a and 56a in the hot plate test (F[4,38] = 0.9254, p > 0.05, and F[4,40] = 1.589, p > 0.05, respectively). As shown in Figure 13 (panel B), STZ slightly increased thermal nociceptive threshold in mice but this effect compared to that of normoglycemic control did not reach statistical significance. In the hot plate test, neither 50a nor 56a was effective.

2.5.7. Effects on Motor Coordination (Rotarod Test). In the rotarod test, the effect of the test compounds on motor coordination of mice was assessed. Compared to the vehicle-treated group, none of the test compounds induced motor deficits in the rotarod test.

3. CONCLUSIONS

Neuropathic pain is a global public health problem and is most frequently caused by chronic, progressive nerve disease after surgery or trauma and viral infections in the course of diabetes or could be induced by chemotherapy. It is worth pointing out that painful diabetic neuropathy is a major complication of diabetes and a cause of increased mortality. Unfortunately, currently used drugs have limited efficacy and patients remain refractory to existing pharmacological treatment. Hence, there is a substantial need for further development of new and effective drugs for NP therapy. Considering the above, the present work describes SAR studies of new functionalized amino acids as inhibitors of GATs, the biological targets in the pathophysiology and pharmacotherapy of NP.

4. METHODS

4.1. Chemistry. Commercially available reagents were purchased from Merck, Aldrich, Acros, or ChemPur and were used without further purification. Solvents for reactions carried out under inert gas (argon), such as tetrahydrofuran (THF) and DCM, were dried, distilled, and collected under argon before use. THF was distilled from a mixture of sodium and benzophenone, while DCM was distilled from calcium hydride. Triethylamine (TEA) was distilled under vacuum before use. Reactions carried out under microwave irradiation used a Discover LabMate (CEM Corporation, USA). Purification of chemical compounds by column chromatography was carried out using Sigma-Aldrich silica gel (mesh 0.063–0.200 mm) as the stationary phase. Reactions were monitored by thin-layer chromatography (TLC) (aluminum sheets precoated with silica gel 60 F254 (Merck)). Compounds were visualized with UV light (254 nm). Additionally, the plates were stained with a 5.5% solution of ninhydrin in n-propanol or a solution of 5% (NH4)2MoO4 and 0.2% Ce(SO4)2 in 5% H2SO4. The retention factor (R) was defined using the following solvent systems: S1 (petrol ether (PE)/EtOAc 7:3, v/v), S2 (PE/EtOAc 1:1, v/v), S4 (n-hexane/ethanol (EtOH)/TEA 7:2.1, v/v), S5 (DCM/methanol (MeOH)/NH4 9:0.5:0.1, v/v/v), S6 (NH2/MeOH/DCM/PE 9:45:10:18, v/v/v/v), S7 (DCM/acetone (Ace) 9:1, v/v), S8 (n-hexane/ethyl acetate (EtOAc) 1:1, v/v), S9 (DCM/MeOH, 95:5, v/v), S10 (chloroform (Cl)/Ace 9:1, v/v), S11 (DCM/ Ace 7:3, v/v), S12 (Cl/Ace 1:1, v/v), S13 (Chl/MeOH/DCl/PE = 640:140:100:25, v/v/v/v). 1H NMR and 13C NMR spectra were recorded on a Varian Mercury VX 300, with 1H at 300.08 MHz and 13C at 75.46 MHz or a JEOL ECA400II or ECX500 at magnetic field strengths of 11.75 T corresponding to 1H and 13C resonance frequencies of 500.16 and 125.77 MHz at ambient temperature (25 °C). Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (J) are reported in hertz (Hz). High-resolution (HR) MS was performed on a Synapt G2-S HDMS (Waters Inc.) mass spectrometer equipped with an electrospray ionization source and q-TOF type mass analyzer. The instrument was controlled, and recorded data were processed using the MassLynx v4.1 software package (Waters Inc.). Purities of the final compounds were determined with a Waters ACQUITY ultraperformance liquid chromatography (UPLC) instrument (Waters, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (ESI-tandem quadrupole).

4.1.1.1. General Procedure for the Synthesis of the 3-Substituted 5-Methylidihydrofuran-2(3H)-one or Dihydrofuran-2(3H)-one De-
rivatives (36, 38, 40, 42, 44, 46) (GP1). A mixture of anhydrous K$_2$CO$_3$ corresponding amine A, C, D, E, or F (1 equiv) and TBAB (0.18 mmol, 0.06 g, 0.01 equiv) in acetonitrile (7 mL) was stirred at 0 °C for 15 min. Then, a solution of 3-bromohydrofuran-2(3H)-one (34) or 3-bromo-5-methylhydrofuran-2(3H)-one (35) (1 equiv) was added dropwise, and stirring continued for 20 h at rt. After the reaction was complete, the precipitate was filtered off, and the filtrate was concentrated under vacuum. The crude product was purified by column chromatography over silica gel.

4.1.1.1.1. 3-[4-[3-(Methylthiophen-2-yl)imidazol-1-yl][methylidene]-piperidin-1-yl]-5-methylxolan-2-one (36). According to GP1 with amine A (17) (0.86 mmol, 0.25 g, 1 equiv), 3-bromo-5-methylhydrofuran-2(3H)-one (34) (0.86 mmol, 0.15 g, 1 equiv), TBAB (0.086 mmol, 27.7 mg, 0.1 equiv), and anhydrous K$_2$CO$_3$ (2.58 mmol, 0.36 g, 3 equiv) were combined in acetonitrile (7 mL). The crude product was purified by column chromatography over silica gel (DCM/Acetone = 9:1) to yield 36 (260 mg, 78%, R$_f$ = 0.65 (Si$_2$) as a yellow oil. Formula C$_9$H$_{10}$N$_2$O$_2$ FW 187.56. ^1H NMR (300 MHz, chloroform-d) δ ppm 1.39 (s, J = 7.58 Hz, 3H (CH$_3$)), 1.39 (s, J = 7.58 Hz, 3H (CH$_3$)), 1.91 (s, J = 7.58 Hz, 3H (CH$_3$)), 1.94 (s, J = 7.58 Hz, 3H (CH$_3$)), 1.96 (s, J = 7.58 Hz, 3H (CH$_3$)). 1.98–2.50 (m, 6H) 2.88–2.89 (m, 3H, 4H) 3.43–3.50 (m, 1H) 4.40–4.52 (m, 1H) 7.19–7.45 (m, 6H (Ar)) 7.63–7.95 (m, 2H (Ar)).

4.1.1.1.2. 3-(4-(9H-Fluoren-9-ylidene)piperidin-1-yl)-5-methylxolan-2-one (40). According to GP1 with amine A (25) (1.17 mmol, 0.29 g, 1 equiv), 3-bromo-5-methylhydrofuran-2(3H)-one (34) (1.17 mmol, 0.21 g, 1 equiv), TBAB (0.12 mmol, 40 mg, 0.1 equiv), and anhydrous K$_2$CO$_3$ (1.51 mmol, 0.68 g, 0.54 equiv) were combined in acetonitrile (10 mL). The crude product was purified by column chromatography over silica gel (DCM/Acetone = 9:1) to yield 40 (289 mg, 71%, R$_f$ = 0.61 (Si$_2$)) as a yellow oil. Formula C$_{20}$H$_{20}$N$_2$O$_2$ FW 361.49. ^1H NMR (300 MHz, chloroform-d) δ ppm 1.39–1.41 (m, 3H) 1.98–2.50 (m, 6H) 2.88–2.89 (m, 3H, 4H) 3.43–3.50 (m, 1H) 4.40–4.52 (m, 1H) 7.19–7.45 (m, 6H (Ar)) 7.63–7.95 (m, 2H (Ar)).

4.1.1.1.3. 3-(4-(9H-Fluoren-9-ylidene)piperidin-1-yl)-5-methylxolan-2-one (42). According to GP1 with amine A (26) (0.22 mmol, 60 mg, 1 equiv), 3-bromo-5-methylhydrofuran-2(3H)-one (34) (0.22 mmol, 40 mg, 1 equiv), TBAB (0.022 mmol, 7 mg, 0.1 equiv), and anhydrous K$_2$CO$_3$ (0.65 mmol, 90 mg, 3 equiv) were combined in acetonitrile (7 mL). The crude product was purified by column chromatography over silica gel (DCM/Acetone = 7:3) to yield 42 (40 mg, 49%, R$_f$ = 0.46 (Si$_2$)) as a yellow oil. Formula C$_{21}$H$_{22}$N$_2$O$_2$ FW 373.49. ^1H NMR (300 MHz, chloroform-d) δ ppm 1.70–1.85 (m, 2H) 2.15 (s, 4H) 2.34–2.54 (m, 4H) 2.76–2.93 (m, 4H) 3.47–3.57 (m, 1H) 3.62–3.76 (m, 3H) 4.30–4.42 (m, 1H) 7.07–7.27 (m, 8H).

4.1.1.1.5. 3-[2-[[Diphenylmethylene]amino]oxyethyl]- (methylamino)-5-methylxolan-2-one (44). According to GP1 with amine F (32) (2.16 mmol, 0.55 g, 1 equiv), 3-(2-bromo-3-methyl-5-methylhydrofuran-2(3H)-one (34) (2.16 mmol, 0.38 g, 1 equiv), TBAB (0.22 mmol, 69.5 mg, 0.1 equiv), and anhydrous K$_2$CO$_3$ (6.48 mmol, 90.9 g, 3 equiv) were combined in acetonitrile (10 mL). The crude product was purified by column chromatography over silica gel (DCM/MeOH = 3:2) to yield 44 (500 mg, 68%, R$_f$ = 0.71 (Si$_2$)) as a yellow oil. Formula C$_{31}$H$_{31}$N$_{2}$O$_3$ FW 532.43. ^1H NMR (300 MHz, chloroform-d) δ ppm 1.34 (J = 6.16 Hz, 3H (CH$_2$CH$_3$)), 1.67 (J = 12.31, 10.52 Hz, 1H (CH$_2$CH$_2$CH$_2$)), 2.07–2.14 (m, 1H (CH$_2$CH$_2$CH$_2$)), 2.38 (3H (NCH$_3$)), 2.85–3.03 (m, 2H (OCH$_2$CH$_2$N)), 3.67–3.79 (m, 1H (NCH)), 4.06–4.23 (m, 1H (CH$_2$)), 4.29–4.36 (m, 2H (OCH$_2$CH$_2$N)) 7.27–7.50 (m, 10H (Ar)).

4.1.1.1.6. 3-[[Diphenylmethylene]amino]oxyethyl]- (methylamino)-oxolan-2-one (46). According to GP1 with amine F (32) (2.95 mmol, 0.75 g, 1 equiv), 3-bromo-5-methylhydrofuran-2(3H)-one (35) (2.95 mmol, 0.52 g, 1 equiv), TBAB (0.30 mmol, 93.2 mg, 0.1 equiv), and anhydrous K$_2$CO$_3$ (8.85 mmol, 1.23 g, 3 equiv) were combined in acetonitrile (10 mL). The crude product was purified by column chromatography over silica gel (DCM/Acetone = 7:3) to yield 46 (520 mg, 52%, R$_f$ = 0.65 (Si$_2$)) as a yellow oil. Formula C$_{33}$H$_{31}$N$_{2}$O$_3$ FW 538.40. ^1H NMR (300 MHz, chloroform-d) δ ppm 2.26–2.31 (m, 1H (CH$_2$CH$_2$)), 2.35–2.55 (m, 4H (Me (CH$_2$CH$_2$))), 2.63–2.74 (m, 2H (CH$_2$N)), 3.49–3.56 (m, 1H (NCH)), 3.64–3.71 (m, 2H (== NOCH$_2$)), 4.25–4.33 (m, 1H (OCH$_2$)), 4.35–4.45 (m, 1H (OCH)), 7.45–7.62 (m, 10H (Ar)).

4.1.1.2. General Procedure for the Synthesis of the 4-Hydroxybutanamide and 4-Hydroxybutanamide Derivatives 37a-c, 39a-c, and 43a-d (GP2). The corresponding 3-substituted 5-methylhydrofuran-2(3H)-one or dihydrofuran-2(3H)-one derivative (0.17 mmol, relevant for the 3-hydroxycholesterol derivative (1.5 equiv), and sodium 2-ethylhexanoate (2.5 equiv) were dissolved in dry THF under Ar and stirred at 66°C for 16 h. Then, the mixture was cooled to rt, and EtOAc (5 mL/1 mmol) and 30% K$_2$CO$_3$ (5 mL/1 mmol) were added. The mixture was stirred at rt for 15 min and was extracted with EtOAc (2 x 5 mL). The combined organic phases were dried over Na$_2$SO$_4$ and concentrated under reduced pressure. The crude product was purified by column chromatography.
and (2RS,4SR)-37b were prepared according to GP2 with 3-[4-[(3-methylthiophen-2-yl)methylidene]piperidin-1-yl]-5-methylxolan-2-one (36) (2.12 mmol, 0.83 g), 1-(2-chlorophenyl)-methanimine hydrochloride (3.18 mmol, 0.56 g), and sodium 2-ethylnitrate (5.34 mmol, 0.95 g) in 10 mL of dry THF. The obtained crude products were purified by column chromatography over silica gel (DCM/Ace = 9:1) to yield (2RS,4SR)-37a (53%, 67.48, 115.29, 115.58, 125.78, 126.13, 127.27, 127.65, 128.10, 128.42, 129.22, 129.33, 130.20, 133.86, 139.91, 139.71, 163.70, 174.81. LCMS m/z [M + H]+ = 487.22. HREMS-ESI m/z [M + H]+ for C18H11NO3S2. 487.2755; found, 487.2758.

4.1.1.2.5. N-(4-Chlorobenzyl)-2-(3-(10,11-dihydro-5H-dibenzo-[a,d]julannulen-5-ylidene)propyl)(methyl)amine)-5-methylidihydrofurano-2(3H)-one (2RS,4SR)-39b and (2RS,4SR)-39c. Compounds (2RS,4SR)-39b and (2RS,4SR)-39c were prepared according to GP2 with 3-[3-(10,11-dihydro-5H-dibenzo-[a,d]julannulen-5-ylidene)propyl](methyl)amine)-5-methylidihydrofurano-2(3H)-one (38) (1.66 mmol, 0.60 g), 1-(4-chlorophenyl)methanimine hydrochloride (2.73 mmol, 0.43 g), and sodium 2-ethylnitrate (4.55 mmol, 0.76 g) in 4 mL of dry THF. The obtained crude products were purified by column chromatography over silica gel (DCM/Ace = 9:1) to yield (2RS,4SR)-39a (53%, 41, 46, 34, 32, 30, 22, 14. HREMS-ESI m/z [M + H]+ for C30H32ClN2O2S2. 486.2755; found, 486.2758.

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(m, 2H (CH2CH2CH2CH2)), 2.05 (s, 3H (ArCH3)), 2.18–2.29 (m, 2H (CH2CHCH2CH2)), 2.32 (s, 3H (NCH3)), 2.44 (br s, 2H (CH2CHCH2CH2)), 2.75 (br s, 1H (ArCH2CH2Ar)), 2.94 (br s, 1H (ArCH2CH2Ar)), 3.12–3.39 (m, 3H (ArCH2ArCH2Ar CH=N), NCH3) 3.67–3.74 (m, 1H (CHOH)), 4.13–4.40 (m, 2H (NCH3)), 5.72 (br s, 1H (CH=N)), 6.27–6.29 (m, 2H (ArH)), 6.72 (br s, 1H (ArH)), 7.15–7.24 (m, 2H (Ar)), 7.20–7.24 (m, 2H (Ar)), 7.74–7.86 (m, 3H (ArH)), 7.92 (br s, 1H (ArH)), 127.5, 127.0, 126.9, 125.8, 125.5, 67.6, 67.4, 53.9, 51.8, 50.3, 43.5, 33.6, 33.2, 31.3, 31.3, 29.7, 29.3, 24.6. HRMS-ESI’ m/z [M + H]+ calc’d for C36H47N2O2 673.3297, found 673.3293.

Compound (2RS,4RS)-4a was purified by preparative TLC with 3-(4-(9H-fluoren-9-yldiene)pentan-5-yl)-5-methyldihydrofuran-2(3H)-one derivative (1 equiv) was heated with the corresponding N-Benzylamine (2 equiv) in dry THF at reflux for 48 h. After the reaction was complete, the mixture was cooled on ice, quenched with 1 M aqueous solution of HCl (1.5 mL), and extracted with dichloromethane (3 x 10 mL). The combined organic fractions were dried over Na2SO4 and evaporated under vacuum. The crude product was purified by column chromatography.

Finally, the purified product was subjected to the following procedure for the synthesis of the 4-Hydroxypentanamide and 4-Hydroxybutanamide Derivatives 47a-c (GP3). These reactions were performed under an argon atmosphere. The relevant 3-substituted S-methylidihydrofuran-2(3H)-one or dihydrofuran-2(3H)-one derivative (1 equiv) was heated with the corresponding N-Benzylamine (2 equiv) in dry THF at reflux for 48 h. After the reaction was complete, the mixture was cooled on ice, quenched with 1 M aqueous solution of HCl (1.5 mL), and extracted with dichloromethane (3 x 10 mL). The combined organic fractions were dried over Na2SO4 and evaporated under vacuum. The crude product was purified by column chromatography.

1.3.1.3.1. N-Benzyl-2-{2-[(diphenylmethylidene)amino]oxyethyl}(methyl)amino)-4-hydroxypentanamide (2RS,4RS)-45 and (2RS,4RS)-45. Compounds (2RS,4RS)-45 and (2RS,4RS)-45 were prepared according to GP3 with 3-{2-[(diphenylmethylidene)amino]oxyethyl}(methyl)amino)-5-methylloxolan-2-one (24) (1.33 mmol, 0.47 g) and N-Benzylamine (1.59 mmol, 0.17 g) in 10 mL of dry THF. The obtained crude products were purified by column chromatography over silica gel (DCM/MeOH = 9:1) to yield (2RS,4RS)-45 (120 mg, 52%, Rf = 0.48 (DCM/MeOH = 9:1) and (2RS,4RS)-45 (30 mg, 13%, Rf = 0.29 (DCM/MeOH = 9:1)) as a yellow oil. The synthetic procedure of the 4-Hydroxypentanamide and 4-Hydroxybutanamide Derivatives 47a-c (GP3) was repeated with the corresponding N-Benzylamine (2 equiv) in dry THF at reflux for 48 h. After the reaction was complete, the mixture was cooled on ice, quenched with 1 M aqueous solution of HCl (1.5 mL), and extracted with dichloromethane (3 x 10 mL). The combined organic fractions were dried over Na2SO4 and evaporated under vacuum. The crude product was purified by column chromatography.

Finally, the purified product was subjected to the following procedure for the synthesis of the 4-Hydroxypentanamide and 4-Hydroxybutanamide Derivatives 47a-c (GP3). These reactions were performed under an argon atmosphere. The relevant 3-substituted S-methylidihydrofuran-2(3H)-one or dihydrofuran-2(3H)-one derivative (1 equiv) was heated with the corresponding N-Benzylamine (2 equiv) in dry THF at reflux for 48 h. After the reaction was complete, the mixture was cooled on ice, quenched with 1 M aqueous solution of HCl (1.5 mL), and extracted with dichloromethane (3 x 10 mL). The combined organic fractions were dried over Na2SO4 and evaporated under vacuum. The crude product was purified by column chromatography.

1.3.1.3.1. N-Benzyl-2-{2-[(diphenylmethylidene)amino]oxyethyl}(methyl)amino)-4-hydroxypentanamide (2RS,4RS)-45 and (2RS,4RS)-45. Compounds (2RS,4RS)-45 and (2RS,4RS)-45 were prepared according to GP3 with 3-{2-[(diphenylmethylidene)amino]oxyethyl}(methyl)amino)-5-methylloxolan-2-one (24) (1.33 mmol, 0.47 g) and N-Benzylamine (1.59 mmol, 0.17 g) in 10 mL of dry THF. The obtained crude products were purified by column chromatography over silica gel (DCM/MeOH = 9:1) to yield (2RS,4RS)-45 (120 mg, 52%, Rf = 0.48 (DCM/MeOH = 9:1) and (2RS,4RS)-45 (30 mg, 13%, Rf = 0.29 (DCM/MeOH = 9:1)) as a yellow oil. The synthetic procedure of the 4-Hydroxypentanamide and 4-Hydroxybutanamide Derivatives 47a-c (GP3) was repeated with the corresponding N-Benzylamine (2 equiv) in dry THF at reflux for 48 h. After the reaction was complete, the mixture was cooled on ice, quenched with 1 M aqueous solution of HCl (1.5 mL), and extracted with dichloromethane (3 x 10 mL). The combined organic fractions were dried over Na2SO4 and evaporated under vacuum. The crude product was purified by column chromatography.

Finally, the purified product was subjected to the following procedure for the synthesis of the 4-Hydroxypentanamide and 4-Hydroxybutanamide Derivatives 47a-c (GP3). These reactions were performed under an argon atmosphere. The relevant 3-substituted S-methylidihydrofuran-2(3H)-one or dihydrofuran-2(3H)-one derivative (1 equiv) was heated with the corresponding N-Benzylamine (2 equiv) in dry THF at reflux for 48 h. After the reaction was complete, the mixture was cooled on ice, quenched with 1 M aqueous solution of HCl (1.5 mL), and extracted with dichloromethane (3 x 10 mL). The combined organic fractions were dried over Na2SO4 and evaporated under vacuum. The crude product was purified by column chromatography.

Finally, the purified product was subjected to the following procedure for the synthesis of the 4-Hydroxypentanamide and 4-Hydroxybutanamide Derivatives 47a-c (GP3). These reactions were performed under an argon atmosphere. The relevant 3-substituted S-methylidihydrofuran-2(3H)-one or dihydrofuran-2(3H)-one derivative (1 equiv) was heated with the corresponding N-Benzylamine (2 equiv) in dry THF at reflux for 48 h. After the reaction was complete, the mixture was cooled on ice, quenched with 1 M aqueous solution of HCl (1.5 mL), and extracted with dichloromethane (3 x 10 mL). The combined organic fractions were dried over Na2SO4 and evaporated under vacuum. The crude product was purified by column chromatography.

Finally, the purified product was subjected to the following procedure for the synthesis of the 4-Hydroxypentanamide and 4-Hydroxybutanamide Derivatives 47a-c (GP3). These reactions were performed under an argon atmosphere. The relevant 3-substituted S-methylidihydrofuran-2(3H)-one or dihydrofuran-2(3H)-one derivative (1 equiv) was heated with the corresponding N-Benzylamine (2 equiv) in dry THF at reflux for 48 h. After the reaction was complete, the mixture was cooled on ice, quenched with 1 M aqueous solution of HCl (1.5 mL), and extracted with dichloromethane (3 x 10 mL). The combined organic fractions were dried over Na2SO4 and evaporated under vacuum. The crude product was purified by column chromatography.

Finally, the purified product was subjected to the following procedure for the synthesis of the 4-Hydroxypentanamide and 4-Hydroxybutanamide Derivatives 47a-c (GP3). These reactions were performed under an argon atmosphere. The relevant 3-substituted S-methylidihydrofuran-2(3H)-one or dihydrofuran-2(3H)-one derivative (1 equiv) was heated with the corresponding N-Benzylamine (2 equiv) in dry THF at reflux for 48 h. After the reaction was complete, the mixture was cooled on ice, quenched with 1 M aqueous solution of HCl (1.5 mL), and extracted with dichloromethane (3 x 10 mL). The combined organic fractions were dried over Na2SO4 and evaporated under vacuum. The crude product was purified by column chromatography.

Finally, the purified product was subjected to the following procedure for the synthesis of the 4-Hydroxypentanamide and 4-Hydroxybutanamide Derivatives 47a-c (GP3). These reactions were performed under an argon atmosphere. The relevant 3-substituted S-methylidihydrofuran-2(3H)-one or dihydrofuran-2(3H)-one derivative (1 equiv) was heated with the corresponding N-Benzylamine (2 equiv) in dry THF at reflux for 48 h. After the reaction was complete, the mixture was cooled on ice, quenched with 1 M aqueous solution of HCl (1.5 mL), and extracted with dichloromethane (3 x 10 mL). The combined organic fractions were dried over Na2SO4 and evaporated under vacuum. The crude product was purified by column chromatography.
Hz, 1H (NHCCH)) 3.96–4.17 (m, 2H (NHCCH)) 4.20–4.34 (m, 3H (OCH2CH2N)) 4.38–4.46 (m, 1H (OHCH)) 7.08–7.14 (m, 2H (Ar)) 7.20–7.47 (m, 13H (Ar)). 13C NMR (126 MHz, chloroform-d) δ ppm 170.0, 157.6, 138.4, 136.1, 133.3, 129.7, 129.1, 129.0, 128.8, 128.7, 128.4, 128.3, 128.0, 127.9, 127.6, 127.6, 127.3, 65.3, 53.4, 43.9, 38.6, 31.5, 29.8, 23.4. HRMS-ESI m/z [M + H]+ calculated for C28H26ClO6 460.2595; found, 460.2593.

4.1.1.3.2. N-Benzyl-2-[(2-[(diphenylmethylidene)amino]oxy)-ethyl](methyl)aminooxacatamidine (47a). Compound 47a was prepared according to GP3 with 3-[(diphenylmethylidene)amino]oxy)-ethyl](methyl)aminooxalan-2-one (46) (1.33 mmol, 0.45 g) and 1-phenylethanamine (1.59 mmol, 0.17 g) in 10 mL of dry THF. The obtained crude product was purified by column chromatography over silica gel (DCM/Ace = 7:3) to yield 47a (352 mg, 56%; Rf = 0.50 (DCM/Ace = 7:3)). Formula C37H33ClNO3S, FW 563.57. 1H NMR (300 MHz, chloroform-d) δ ppm 1.81–1.94 (m, 2H (CH2CH2NH2) 2.28 (s, 3H (Me) 2.79 (s, t, J = 5.39 Hz, 2H (CH2N)) 3.28–3.35 (m, 1H (NHCCH)) 3.52–3.62 (m, 1H (CH2OH)) 3.78–3.87 (m, 1H (CH2OH)) 4.19 (dd, t, J = 14.88, 6.41 Hz, 2H (CH2O)) 4.23–4.31 (m, 2H (NHCH2)) 7.07–7.13 (m, 13H (Ar)) 7.18–7.47 (m, 13H (Ar)) 7.78 (t, t, J = 5.90 Hz, 1H (CONH)). 13C NMR (300 MHz, chloroform-d) δ ppm 14.20, 27.39, 28.39, 43.02, 53.37, 60.40, 61.68, 67.48, 72.43, 127.21, 127.33, 127.83, 128.13, 128.30, 128.93, 129.06, 129.49, 133.13, 136.14, 138.26, 157.15, 174.07. HRMS-ESI m/z [M + H]+ calculated for C29H27ClNO3S 504.2644; found, 504.2647.

4.1.1.3.3. N-g-(3,4-Dichlorophenyl)methyl]-2-[(2-[(diphenylmethylidene)amino]oxy)-ethyl](methyl)aminooxalan-2-one (47b). Compound 47b was prepared according to GP3 with 3-[(diphenylmethylidene)amino]oxy)-ethyl](methyl)aminooxalan-2-one (46) (1.33 mmol, 0.45 g) and 1-(3-chlorophenyl)ethanamine (1.59 mmol, 0.23 g) in 10 mL of dry THF. The obtained crude product was purified by column chromatography over silica gel (DCM/Ace = 7:3) to yield 47b (223 mg, 35%; Rf = 0.52 (DCM/Ace = 7:3)). Formula C36H31Cl2NO3, FW 533.48. 1H NMR (300 MHz, chloroform-d) δ ppm 1.82–1.92 (m, 2H (CH2CH2NH2) 2.28 (s, 3H (Me) 2.80 (s, t, J = 5.51 Hz, 2H (CH2N)) 3.28–3.36 (m, 1H (NHCCH)) 3.50–3.60 (m, 1H (CH2OH)) 3.81 (dt, t, J = 11.09, 4.58 Hz, 1H (CH2OH)) 4.18–4.34 (m, 4H (NHCH2)) 7.10–7.49 (m, 14H (Ar)) 7.86 (t, t, J = 5.90 Hz, 1H (CONH)). 13C NMR (300 MHz, chloroform-d) δ ppm 27.22, 29.69, 34.21, 58.14, 53.38, 61.68, 67.31, 72.57, 126.91, 127.81, 128.08, 128.25, 128.57, 128.91, 129.02, 129.39, 129.47, 133.09, 133.21, 135.54, 136.12, 175.18, 174.31. HRMS-ESI m/z [M + H]+ calculated for C36H30Cl2NO3S 540.2584; found, 540.2586.

4.1.1.4. General Procedure for the Synthesis of N-Benzyl-4-(3,4-oxo-2-yl)-4-diphenylmethylidenepeperidin-1-ylbutanamide derivatives (49a,b). According to GP3, amide 48a (1.35 mmol, 0.59 g) amine F (diphenylmethane O-(2-(methylamino)ethyl) oxime (32)) (1.76 mmol, 0.44 g, 1 equiv) in acetonitrile (10 mL). Then, the relevant N-Benzyl-2-bromo-4-(1,3-dioxoisoindolin-2-yl)butanamide derivative (48a,b) (1.76 mmol, 1 equiv) was added, and the reaction mixture was stirred at reflux for 24 h. Then, the precipitate was filtered, the filtrate was concentrated under vacuum, and the product was purified by column chromatography.

4.1.1.4.1. N-(2-Chlorophenyl)methyl]-4-(1,3-dioxo-2,3-dihydro-1H-isoindol-2-yl)-2-[(2-[(diphenylmethylidene)amino]oxy)-ethyl](methyl)aminooxalan-2-one (49a). According to GP4, amide 48b (1.35 mmol, 0.63 g) amine F (diphenylmethane O-(2-(methylamino)ethyl) oxime (32)) (1.35 mmol, 0.34 g), KI (1.22 mmol, 0.20 g), and anhydrous K2CO3 (3.11 mmol, 0.43 g) were combined in acetonitrile (15 mL). The obtained crude product was purified by column chromatography over silica gel (PE/EtOAc = 1:1) to yield 49a (346 mg, 42%, Rf = 0.49 (PE/EtOAc (1:1)) as a yellow oil. Formula C36H32Cl2N4O4, FW 643.57. 1H NMR (300 MHz, chloroform-d) δ ppm 1.84–1.97 (m, 1H (CH2CH2NH)) 2.14–2.23 (m, 1H (CH2CH2NH)) 2.32 (s, 3H (Me) 2.80 (t, J = 5.62 Hz, 2H (CH2N)) 2.67 (d, t, J = 13.91, 7.76, 6.54 Hz, 1H (NHCCH)) 3.85–3.99 (m, 2H (OHCH2)) 4.25 (s, t, J = 5.26 Hz, 2H (NHCH2)) 6.91 (dd, t, J = 8.21, 2.05 Hz, 1H (Ar) 7.10 (d, J = 2.05 Hz, 1H (Ar) 7.20–7.44 (m, 11H (Ar)) 7.44–7.50 (m, 1H (CONH)) 7.66–7.74 (m, 2H (phenylamide)) 7.78–7.86 (m, 2H (phenylamide)).

4.1.1.5. General Procedure for the Synthesis of 2-Substituted 4-Aminobutanamide Derivatives (50a,b) (GP5). Hydrazide hydrate (2 equiv) was added to a suspension of a 2-substituted -4-phenylbutanamic acid derivative (1 equiv) in ethanol (10 mL). The solution was stirred at 60 °C for 2 h and at rt for 5 h. Then, the precipitate was filtered and washed with DCM (5 mL). The filtrate was evaporated, and the product was extracted two times with 8 mL of DCM. The combined organic fractions were dried over Na2SO4, and the obtained product was purified by column chromatography.
4.1.1.5.2. 4-Amino-N-[[3,4-dichlorophenyl]methyl]-2-[[2-[[[diphenylmethyldiene]amino]oxy]ethyl]methyl]amino]butanamide (50b). According to GP5, N-(3,4-dichlorobenzoyl)-1-(3,4-dioxoisindolin-2-yl)-2-[[[[diphenylmethyldiene]amino]oxy]ethyl][methyl]amino]butanamide (49b) (1 mmol, 0.64 g) and hydrazine hydrate (2 mmol, 0.11 g) were combined in ethanol (5 mL). The obtained crude product was purified by column chromatography over silica gel (EtOAc/MeOH = 8 : 2 → 25% in 95:4:DCM/CHCl3/MeOH = 9:5:4:DCM/PE = 9:4:1.89 (m, 5H (CH2=CH(CH3)2) 3.13-3.33 (2H, (CH2)3), 3.33-3.45 (m, 1H (NCH2)), 4.16-4.43 (m, 4H (NHCCH2CH2O)); 6.94 (br s, 1H (CONH)) 7.14-7.24 (m, 14H (Ar)) 7.75 (t, J = 6.03 Hz (CONH)). 13C NMR (300 MHz, chloroform-d) δ ppm 23.25, 23.44, 38.53, 39.90, 41.10, 53.42, 66.52, 66.52, 72.54, 126.87, 127.80, 128.40, 128.06, 128.24, 128.24, 128.54, 128.87, 128.88, 129.03, 129.03, 129.43, 129.53, 133.14, 133.21, 135.68, 136.11, 137.11, 170.30, 173.88. HRMS-ESI”m/z [M + H]+ calculated for C26H29ClN2O2S2, 501.1034. Found: 501.1014. 4.1.1.6. General Procedure for the Synthesis of 2-Substituted 4-Acetamidobutanamides Derivatives (51a-b, GP6). A mixture of acetic acid (2 equiv) and NaN3 (0.11 g) in DMF for 16 h, extraction with DCM was carried out. The obtained crude product was purified by column chromatography over silica gel (DCM/MeOH = 7:3) to yield 54c (100 mg, 36%, Rf = 0.17) as a yellow solid. 

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Silica gel (DCM/Acetone = 7:3) to yield 55a ([1 mmol, 0.32 mg), DIPEA (1 mmol, 174 μL), DCM 5 mL. The obtained crude product was purified by column chromatography over silica gel (DCM/Acetone = 7:3) to yield 55a ([15 mg, 30%, Rf = 0.66 and 0.42 (S1b)). ^1H NMR (chloroform-d): δ ppm 7.35 (s, 1H, CHAr), 7.31–7.28 (m, 4H, CHAr), 7.07–7.04 (m, 2H, CHAr), 6.92–6.89 (m, 1H, CHAr = CHAr), 5.52–5.49 (m, 2H, CH2Ar), 4.21–4.18 (m, 2H, CH2Ar), 2.77–2.74 (m, 2H, CH2Ar), 2.19–2.16 (m, 2H, CH2Ar = CHAr), 2.01–1.98 (m, 3H, CH3–N) ppm. OH proton was not detected. ^13C NMR (chloroform-d): δ ppm 171.25 (CAr), 143.81 (PhC=C), 143.30 (C1a), 142.62 (C1b), 131.41 (C1d), 130.91 (Ar=CH=CHAr), 130.89 (C7, 1H, NAr), 130.43 (C1e, 1H, NAr), 129.81 (C9, 1H, NAr), 129.42 (C8, 1H, NAr), 128.50 (C10, 1H, NAr), 127.72 (C11, 1H, NAr), 126.98 (C12, 1H, NAr), 125.60 (C13, 1H, NAr), 124.29 (C14, 1H, NAr), 123.19 (C15, 1H, NAr), 121.27 (C16, 1H, NAr), 119.73 (C17, 1H, NAr), 118.72 (C18, 1H, NAr). HRMS-ESI+ m/z ([M + H]+): 420.2 (CHAr), 219.1 (CHAr = CHAr), 171.2 (CH2Ar = CHAr), 161.9 (Ar=CH=CHAr), 159.03 (C7, 1H, NAr), 158.09 (C8, 1H, NAr), 157.97 (C9, 1H, NAr), 157.65 (C10, 1H, NAr), 157.33 (C11, 1H, NAr), 157.02 (C12, 1H, NAr), 156.66 (C13, 1H, NAr), 156.40 (C14, 1H, NAr), 156.17 (C15, 1H, NAr), 155.76 (C16, 1H, NAr), 155.44 (C17, 1H, NAr), 155.13 (C18, 1H, NAr).
The synthesized compound was purified by column chromatography over silica gel (DCM/ acetone = 7:3) to yield 58 (55 mg, 30%, Rf = 0.33 and 0.45 (DCM/acetone 7:3) as a yellow oil. Formula C30H28N2O2 FW: 452.68. 1H NMR (300 MHz, chloroform-d) δ ppm 7.57–7.53 (m, 2H), 7.47–7.43 (m, 2H), 7.36–7.33 (m, 1H), 5.85–5.60 (m, 1H), 4.50 (d, J = 9.23 Hz, 1H), 4.42 (s, 1H), 3.82–4.05 (m, 2H), 3.67–3.83 (m, 1H), 3.18–3.59 (m, 2H), 2.76–3.04 (m, 2H), 2.57 (s, 1H), 2.38–2.49 (m, 1H), 1.20–1.50 (m, 20H). 4.1.1.7.17. N-Benzyl-2-[(4,10,11-diaryl-5H-dibenzo[a,d] annulene-5-ylidenepiperidin-1-yl)-3-hydroxypropamidine (59a). The synthesis was done according to GP7 with amine E (26) (0.70 mmol, 290 mg), amide 53a (0.58 mmol, 150 mg), TBAB (0.06 mmol, 19 mg), DIPEA (0.58 mmol, 100 μL), and DMF (5 mL). The obtained crude product was purified by column chromatography over silica gel (DCM/acetone = 7:3) to yield 59a (105 mg, 40%, Rf = 0.22 and 0.54 (DCM/acetone 7:3) as a yellow oil. Formula C41H33N3O2 FW: 587.72. 1H NMR (300 MHz, chloroform-d) δ ppm 7.35–7.27 (m, 2H), 7.24–7.17 (m, 2H), 7.15–7.07 (m, 2H), 7.07 (d, J = 7.03 Hz, 2H), 4.44 (d, J = 5.27 Hz, 3H), 3.21–3.43 (m, 3H), 3.06–3.18 (m, 2H), 2.77–2.30 (m, 3H), 2.49–2.72 (m, 6H).
4.1.1.7.19. N-Benzyl-2-bromopropanamide (62). The synthesis was done according to GP8 N-benzylamine (8.8 mmol, 962 μL) with 2-bromopropionic acid (60) (8 mmol, 724 μL), TEA (9.6 mmol, 1.24 mL), T3P (8 mmol, 4.76 mL), and DCM (32 mL) to yield 62 (1.64 g, 85%, Rf = 0.65 (S)). Formula: C7H11BrNO (241.01 g/mol).

4.1.1.7.20. N-Benzyl-2-bromobutanamide (63a). The synthesis was done according to GP8 N-benzylamine (8.8 mmol, 962 μL) with 2-bromopropionic acid (61) (8 mmol, 850 μL), TEA (9.6 mmol, 1.24 mL), T3P (8 mmol, 4.76 mL), and DCM (32 mL) to yield 63a (1.63 g, 80%, Rf = 0.60 (S)). Formula: C8H12BrNO (255.01 g/mol).

4.1.1.7.21. 2-Bromo-N-(2-chlorobenzyl)butanamide (63b). The synthesis was done according to GP8 N-benzylamine (8.8 mmol, 1062 μL) with 2-bromopropionic acid (61) (8 mmol, 850 μL), TEA (9.6 mmol, 1.24 mL), T3P (8 mmol, 4.76 mL), and DCM (32 mL) to yield 63b (1.9 g, 82%, Rf = 0.7 (S)). Formula: C11H13BrClNO (288.98 g/mol).

4.1.1.8. General Procedure for the Synthesis of Substituted Propanamide Derivatives 64a,b, 65, 66a,b, 67, 68. The general procedure is corresponding to the GP7 method (general procedures for the synthesis of 2-substituted 3-hydroxypropanamide derivatives described in section 4.1.1.7).

4.1.1.8.1. N-Benzyl-2-(4-(bis(3-methylthiophen-2-yl)ethylenyl)piperidin-1-yl)propanamide (64a). The synthesis was done according to GP7 with amine A (17) (0.48 mmol, 135 mg), amide 63a (0.4 mmol, 102 mg), TBAB (0.04 mmol, 0.12 mg), TEA (0.4 mmol, 55 μL), DCM 0.5 mL. The obtained crude product was purified by column chromatography over silica gel (DCM/Ace = 3:7) to yield 59c (101 mg, 55%, Rf = 0.15 (S5)).

4.1.1.8.2. 2-((3-Methylthiophen-2-yl)ethylenyl)piperidin-1-yl)-N-(2-chlorobenzyl)butanamide (64b). The synthesis was done according to GP7 with amine A (17) (0.48 mmol, 135 mg), amide 63b (0.4 mmol, 115 mg), TBAB (0.04 mmol, 0.12 mg), TEA (0.4 mmol, 55 μL), DCM 0.5 mL. The obtained crude product was purified by column chromatography over silica gel (DCM/Ace = 7:3) to yield 64b (119 mg, 60%, Rf = 0.25 (S5)).

4.1.1.8.3. N-Benzyl-2-((di(3-methylthiophen-2-yl)ethylenyl)methyl)(amino)propanamide (65). The synthesis was done according to GP7 with amine F (32) (0.48 mmol, 122 mg), amide 63a (0.4 mmol, 102 mg), TBAB (0.04 mmol, 0.12 mg), TEA (0.4 mmol, 55 μL), DCM 0.5 mL. The obtained crude product was purified by column chromatography over silica gel (DCM/Ace = 7:3) to yield 65 (191 mg, 55%, Rf = 0.3 (S5)).
mmol, 55 μL). DCM 0.5 mL. The crude product was purified by column chromatography over silica gel (DCM/Acetone = 7:3) to yield 68 (90 mg, 50%, Rf = 0.35 (Si)). 1H NMR (300 MHz, chloroform-d) δ ppm 0.97 (t, J = 7.33 Hz, 3H, CH3(CH2)3) 1.56–1.87 (m, 3H, thiop(CH2)3) 1.88–1.92 (m, 3H, thiop(CH2)3) 1.98–2.04 (m, 3H, NCH3) 2.18 (s, 2H, CH=CHMe) 2.28 (q, J = 7.03 Hz, 2H) 2.54–2.69 (m, 2H, CH(CH3)2) 2.78 (m, 1H, CH=N=CH2) 2.95 (d, J = 18.7 Hz, 1H, NHCONH) 3.43–4.55 (m, 2H, NHCH2Ar) 5.89 (t, J = 7.33, 1H, H) 6.74 (d, J = 5.27 Hz, 1 H, thiop) 6.83 (d, J = 5.27 Hz, 1H, H, thiop) 6.98–7.08 (m, 7H, thiop) 7.15–7.38 (m, 6H, Ar, thiop) 7.44 (br s, 4H) 7.62 (br s, 1 H, NHCO). 13C NMR HRMS-ESI+ m/z [M + H]+ calcd for C20H27NO2S2, 378.1556; found, 378.1556.

4.1.1.10. General Procedure for the Synthesis of Ethyl 2-Substituted Propanoate 71, 73 and Benzyl 2-Substituted Butanoate 72 (GP11). The corresponding amine B (5.27 equiv), a relevant ester 69 or 70 (1 equiv), and K2CO3 (3.5 equiv) were transferred directly from the templates.

4.1.1.11. General Procedure for the Hydrolysis of Ethyl and Benzyl 2-Substituted Butanoate 71 and 73 and 74 (GP12). An aqueous 10 wt % NaOH (0.15 g/1.5 mL) solution was added to compounds 71 and 73 (0.3 mmol) and stirred for 4.5 h at 35 °C. The aqueous phase was acidified to pH = 3 with 3q HC1 (1 M) and extracted with DCM twice. The combined organic phases were dried over anhydrous Na2SO4, filtered, and then concentrated to give product.

4.1.1.11.1. 4.1.1.11.1. N-(5H-Dibenzo[a,d][7]annulen-5-ylidene)propyl)-N-methylalanine (74). The synthesis was done according to GP12 with ethyl N-(5H-dibenzo[a,d][7]annulen-5-ylidene)propyl)-N-methylalanine (71) (108 mg, 0.3 mmol) in 2 mL of 10 wt % NaOH to give the oil (quantitative) (100 mg). 1H NMR (300 MHz, chloroform-d) δ 7.20 (d, J = 2.34 Hz, 2H), 7.00–7.20 (m, 8H), 6.99–7.21 (m, 8H), 6.73–6.77 (m, 2H), 5.37 (t, J = 5.28 Hz, 1H), 3.14–3.35 (m, 1H), 2.53–3.03 (m, 3H), 2.33 (br s, 4H), 1.10–1.22 (m, 3H). COOH proton was not detected. HRMS-ESI+ m/z [M + H]+ calcd for C21H23N2O3, 334.1870; found, 334.1805.

4.2. In Vitro Activity. 4.2.1. [3H]GABA Uptake Assay. The inhibitory activities of the synthesized compounds were determined from [3H]GABA uptake assays with mGAT1, mGAT2, mGAT3, and mGAT4 as described previously.19,50 and all compounds were tested at a screening concentration of 100 μM.

4.2.2. MS Binding Assays. MS binding assays for mGAT1 were performed as described earlier.19 Inhibition of mGAT1 binding by the synthesized compounds was determined in MS binding assays at a screening concentration of 100 μM and analyzed by LC–ESI-MS/MS.

4.3. Molecular Modelling. 4.3.1. Docking Studies. For the docking studies, we used models of human GAT-1, GAT-2, and GAT-3 that were selected in our previous work.51 They were built with the SWISS-MODEL server based on the 4XP9 template from the Protein Data Bank (PDB). For BGT-1 we decided to apply the extra model built on the same template, according to previously described procedure.51 In this model, the nonhelical fragment of TM10 (residues 455–459) was additionally optimized using the MyLoop class in the Modeller program. From among 100 refined models, the best one was selected according to the QMEAN score. For each type of GABA transporter we used sequence alignment generated automatically by SWISS-MODEL. The N- and C-termini were omitted because of their low homology. Sodium and chloride ions were transferred directly from the templates.

The ligand 3D structures were created in the Maestro program. Ionization states were predicted under physiological conditions (pH 7.4) using the Epik and Marvin programs. Ligands were optimized in the LigPrep module. All possible stereoisomers for each ligand were generated. Models were prepared with Protein Preparation Wizard using the default settings.

The most active compound representatives were initially docked into the models of each type of GABA transporter using the induced-fit docking protocol available in the Schrödinger Suite. The box center was defined by residues PHE294, TYR140, TYR452, and ARG69 in GAT-1 and the corresponding amino acids in BGT-1, GAT-2, and GAT-3. The box size was 10 Å × 10 Å × 10 Å. The obtained complexes were then visually inspected in terms of the created interactions, frequency, and score of the poses as well as their coherence between the different types of GATs. After selection of the best optimized models, all studied compounds were docked into the models using the GLIDE program and the final conformation of the models was selected based on ligand pose coherence. The grid center in GLIDE was set as the centroid of the ligand from the complex, and the inner box size was 15 Å × 15 Å × 15 Å. The OPLS2005 force field was applied during grid generation as well as GLIDE and IFP docking.

4.3.2. Molecular Dynamics. MD simulations were performed with NAMD using the CHARMM36m force field. Before simulations, all models were prepared in the membrane using the OPM server, and input files for NAMD were prepared with the CHARMM-GUI online server. The protein–ligand complexes were embedded in a 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) membrane and solvated with TIP3P water molecules. The system size was 100 Å × 100 Å. A water pore for each complex was generated. Sodium and chloride ions (0.15 M NaCl) were added to provide standard

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physiological ionic strength. The system was equilibrated via a six step protocol recommended by CHARMM-GUI for the NAMD program. MD simulations were run at 303.15 K with a time step of 2 fs and a total duration of 10 ns. The intervals for both the energy and trajectory recordings were 10 ps. The results were analyzed with the VMD program.

4.4. Hepatotoxicity and Cytotoxicity. Hepatotoxicity and cytotoxicity were estimated according to previously described protocols, using the hepatoma HepG2 (ATCC HB-8065) and human embryonic kidney HEK-293 (ATCC CRL1573) cell lines, respectively. In brief, cells were seeded in 96-well plates at a density of 0.7 × 10^4 and cultured at 37 °C in an atmosphere containing 5% CO2. Next, the compounds were added and investigated in quadruplicate at concentrations ranging from 0.1 to 100 μM for 72 h. The antiproliferative drug DX was used as the reference. The CellTiter 96 AQueous nonradioactive cell proliferation assay (MTS) purchased from Promega (Madison, WI, USA) was used for the determination of cell viability. The absorbance at 492 nm was measured using an EnSpire microplate reader (PerkinElmer, Waltham, MA, USA).

4.5. In Vivo Evaluation. 4.5.1. Materials and Methods. 4.5.1.1. Animals and Housing Conditions. Behavioral experiments were carried out at the Department of Pharmacodynamics, Faculty of Pharmacy, Jagiellonian University Medical College, Krakow. Tests were performed between 9 a.m. and 2 p.m. All experimental in vivo procedures were approved by the second Local Ethics Committee in Krakow (Approval Numbers 32/2018 and 508/2021). The treatment of animals was in full accordance with the ethical standards laid down by both Polish and EU regulations (Directive 2010/63/EU). To avoid potential bias in data recording, the investigators who were involved in behavioral assays were blinded to the experimental groups. Adult male albino Swiss (CD-1) mice weighing 18–22 g were supplied by the Animal Breeding Farm of the Jagiellonian University Faculty of Pharmacy. Before the in vivo tests, the mice were kept in groups of 10 in standard plastic cages. Bedding material (Transwior, Poland) was at least 2 cm deep to allow the mice to dig, and animals were housed under controlled laboratory conditions (room temperature of 22 ± 2 °C; light/dark (12:12) cycle, lights on at 8 a.m., humidity 50 ± 10%, and free access to food (Murigran, Agropol, Poland) and tap water). Experimental groups consisted of 8–10 animals/dose. For behavioral tests, the mice were selected randomly after completion of the assays, the mice were euthanized by cervical dislocation.

4.5.1.2. Chemicals Used in the in Vivo Tests. Before the in vivo tests, the test compounds were suspended in 1% Tween 80 (Baxter, Poland). The compounds were then administered intraperitoneally. The dose of 30 mg/kg of each compound was the starting dose, and if activity was observed in the pain tests, a dose of 10 mg/kg was also tested. The test compounds were administered only once daily on days 1 and 7 of oxaliplatin- or paclitaxel-induced neuropathy. In STZ-treated mice, test compounds were administered 21 days after STZ injection. Control mice used in oxaliplatin and paclitaxel NP models were injected with an appropriate amount of vehicle (0.9% saline). Oxaliplatin was purchased from Activate Scientific GmbH (Germany). Paclitaxel and STZ were purchased from Sigma-Aldrich (Poland).

4.5.1.3. Induction of Neuropathy and NP. For pain studies, oxaliplatin was dissolved in 5% glucose solution (Polfa Kutno, Poland). The dose of oxaliplatin used to induce peripheral neuropathy (10 mg/kg, intraperitoneal injection) was chosen on the basis of previous research and available literature data. Doses of both paclitaxel and STZ used for the induction of neuropathy were selected based on our previous research. To induce neuropathy, paclitaxel was used at a dose of 18 mg/kg. It was prepared by dissolving in ethanol (100% v/v); Polskie Odczynniki Chemiczne, Gliwice, Poland) at 10% of the final desired volume and volume for 2% hydroxypropyl-β-cyclodextrin (10% of the final volume) was then added, and the mixture was vortexed for the next 10 min. Prior to injection, ice-cold physiological saline (80% of the final volume) was added to make up a final volume and the solution was maintained on ice during dosing. To induce type I diabetes, mice were intraperitoneally injected with STZ (a single injection of STZ, 200 mg/kg) dissolved in 0.1 N citrate buffer. Age-matched control mice received an equal volume of citrate buffer. Blood glucose level was measured 1 day before (referred to as “day 0”) and repeatedly 1, 2, and 3 weeks after STZ injection using a blood glucose monitoring system (Accu-Chek Active, Roche, France). Blood samples for measurement of glucose concentration were obtained from the tail vein of the mice. The animals were considered as diabetic when their blood glucose concentration exceeded 300 mg/dL, and only these mice (diabetic mice) were used in subsequent pain tests.

4.5.1.4. Assessment of Tactile (Mechanical) Allodynia (von Frey Test). The ability of the test compounds to attenuate tactile allodynia caused by oxaliplatin, paclitaxel, and STZ was assessed using the von Frey test. For this purpose, 3 h after oxaliplatin or paclitaxel and 21 days after STZ injection, the predrug paw withdrawal threshold was measured for each mouse. Then, the test compounds were administered, and 1 h later, the postdrug paw withdrawal threshold was collected for each animal. This part of the experiment aimed to establish the effect of treatment on early phase (acute) pain hypersensitivity induced by cytotoxic drugs. Additionally, in the oxaliplatin model and the paclitaxel model, to assess the effect of test compounds on late phase tactile allodynia, 7 days later, measurements of the predrug and postdrug paw withdrawal thresholds were made in a similar manner to the measurements performed during early phase neuropathy. At this stage of the experiment, there was no additional oxaliplatin/paclitaxel prevention administration. An electronic von Frey unit (Bioseb, France) was used to assess the mechanical nociceptive threshold (tactile allodynia) in mice. This device has a single flexible filament that applies increasing force (from 0 to 10 g) against the plantar surface of the hind paw of each mouse. In the von Frey test, the paw withdrawal response of the animals automatically turns off the stimulus, and the mechanical pressure that evokes this response is recorded. On the day of the experiment, the mice were placed individually in test compartments with a wire mesh bottom and left there for 1 h of habituation. Subsequently, to obtain baseline values, each mouse was tested 3 times alternately in each hind paw. Then, the test compounds were administered, and 1 h later, 3 additional measurements were taken and averaged to obtain the mean postdrug values for each mouse.

4.5.1.5. Assessment of Cold Nociceptive Threshold (Cold Plate Test). A cold plate apparatus (hot/cold plate, Bioseb, France) set at 2.5 °C was used to assess the effect of treatment on cold hyperalgesia in oxaliplatin-treated mice. The cold plate test was conducted immediately after the von Frey test. Three hours after oxaliplatin administration, the animals were placed on a cold plate apparatus, and predrug latencies to pain reaction (i.e., lifting, biting, shaking of hind paws, jumping, movement deficits, or withering response) were collected. Finally, the test compounds were injected, and 1 h later, the postdrug latencies to pain reaction were measured. In this assay, a cutoff time of 60 s was established to avoid potential thermally induced damage to the paw tissues, and animals not responding within 60 s were removed from the apparatus and assigned a score of 60 s.

4.5.1.6. Assessment of Heat Nociceptive Threshold (Hot Plate Test). Thermal (heat) nociceptive threshold was assessed in the hot plate test as previously described. First, baseline (predrug) latencies to pain reaction were established for each mouse. Then, the mice were treated intraperitoneally with either the test compound or vehicle. Sixty minutes later the animals were placed on the hot plate apparatus again (hot/cold plate, Bioseb, France). This apparatus has an electrically heated surface and is supplied with a temperature-controller that maintains the temperature at 55 °C. The time until the animal licked its hind paws or jumped was recorded by means of a stop-watch. In this assay a cutoff time was established (60 s) to avoid tissue damage, and the mice not responding within 60 s were removed from the apparatus and assigned a score of 60 s.

4.5.1.7. Rotarod Test. Before the rotarod test, the experimental animals underwent 3 d of training on the rotarod apparatus (rotarod apparatus, May Commat R0711, Turkey; rod diameter 2 cm) that
rotated at a fixed speed of 18 rotations per minute (rpm). During this training session, the mice were placed on the rotating rod for 3 min with an unlimited number of trials. The proper test was performed 24 h after the last training session. Sixty minutes after administration of the test compounds or vehicle, the mice were tested on rods that revolved at 6, 18, and 24 rpm. Motor deficits in the mice were defined as their inability to remain on the rotarod apparatus for 1 min. The results are expressed as the mean time spent on the rotarod.68

4.5.1.8. Data Analysis. Data analysis of the in vivo results was performed using GraphPad Prism software (version 8.0, CA, USA). Numerical results obtained in behavioral tests are expressed as their inability to remain on the rotarod apparatus for 1 min. The results are expressed as the mean ± SEM. Statistical analysis was performed using the Shapiro–Wilk normality test, followed by one-way analysis of variance (ANOVA) and Tukey’s post hoc comparison (NP models). One-way ANOVA and Dunnett’s post hoc comparison were used to test differences between the drug-treated groups and the control group in the rotarod test. A value of p < 0.05 was considered significant.

■ ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.1c00351.

Supporting Information
4.5.1.8. Data Analysis. Data analysis of the in vivo results was performed using GraphPad Prism software (version 8.0, CA, USA). Numerical results obtained in behavioral tests are expressed as the mean ± SEM. Statistical analysis was performed using the Shapiro–Wilk normality test, followed by one-way analysis of variance (ANOVA) and Tukey’s post hoc comparison (NP models). One-way ANOVA and Dunnett’s post hoc comparison were used to test differences between the drug-treated groups and the control group in the rotarod test. A value of p < 0.05 was considered significant.

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Author Contributions
P.Z. and K.K. designed and coordinated the study and analyzed the data. B.G., P.Z., and K.S. wrote the manuscript. B.G., P.Z., K.M., and G.M. synthesized the compounds. G.C.H. coordinated [3H] GABA uptake assay and MS binding assays. K.L. and M.B. performed molecular modeling. G.L. performed in vitro studies (hepatotoxicity and cytotoxicity). K.S. and A.R. performed in vivo pharmacological evaluation (mouse models of neuropathic pain). B.M., K.S., K.T.W., and G.C.H. corrected the manuscript. All the authors contributed to and approved the final manuscript.

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Notes
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■ ABBREVIATIONS

GABA, γ-aminobutyric acid; CNS, central nervous system; GABA<sub>A</sub>, γ-aminobutyric acid type A receptor; GABA<sub>B</sub>, γ-aminobutyric acid type B receptor; GABA<sub>C</sub>, γ-aminobutyric acid type C receptor; GAT, GABA transporter protein; SLC6, solute carrier family; BGT1, betaine/GABA transporter 1; IUPHAR, International Union of Basic and Clinical Pharmacology; GAT1, GABA transporter 1; GAT2, GABA transporter 2; RMSD, root-mean-square deviation; GAT3, GABA transporter 3; NP, neuropathic pain; FDA, U.S. Food and Drug Administration; BOC, tert-butyloxycarbonyl protecting group; TEA, triethylamine; THF, tetrahydrofuran; DCM, dichloromethane; STZ, streptozotocin.

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