New insights into the mechanism of action of Trofinetide: An exploratory in silico study.

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Abstract

Keywords:

Introduction

Rett syndrome (RTT) is a rare and complex genetic neurodevelopmental disorder that primarily affects girls, with an estimated prevalence of 1 in 10,000 to 15,000 live births [1]. It is characterized by a period of apparently normal development, followed by a progressive regression of motor, social, and communication skills. Critical symptoms of the disease include the loss of voluntary use of the hands and the appearance of repetitive movements, gait disturbances, severe cognitive impairment, respiratory irregularities, seizures, dysautonomia, and metabolic alterations such as dyslipidemia, insulin resistance, or mitochondrial dysfunction [2–4]. These manifestations reflect a multisystemic affectation that impacts both the central nervous system as well as the endocrine and bioenergetic systems. The course of the disease leads to a progressive deterioration that compromises the quality of life of patients, who require permanent medical attention and specialized care [5]. Although life expectancy can extend into adulthood, it varies depending on the phenotype, severity of symptoms, and quality of medical management [6].

The main cause of RTT is the presence of mutations in the MECP2 gene, located on the X chromosome, which encodes the methyl CpG-binding protein 2, a transcription regulator essential for neuronal maturation and synaptic plasticity [7]. There are also atypical variants of the syndrome that occur less frequently and are due to mutations in the CDKL5 or FOXG1 genes [8]. The disease usually appears between 6 and 18 months of age and causes lifelong disability, with no curative treatment currently available. Since in its early stages, symptoms can be confused with disorders such as autism, early diagnosis represents a major clinical challenge.

Until recently, the therapeutic approach to RTT was limited to disease control through the use of drugs and physical, occupational, and speech therapies. These actions are primarily aimed at mitigating the most disabling clinical manifestations, such as motor disorders, epileptic seizures, respiratory, digestive, and autonomic disorders, as well as behavioral

problems. In this context, the use of anticonvulsants, anxiolytics, serotonergic modulators, beta-blockers, as well as interdisciplinary functional support strategies, is common [1].

In 2023, the FDA approved the drug Trofinetide, marking a milestone by becoming the first targeted therapy for this disease [9]. Trofinetide is an analogous of Glypromate (Glycine-Proline-Glutamic Acid or simply GPE) peptide derived from the N-terminus of insulin-like growth factor 1 (IGF-1) and was designed to cross the blood-brain barrier and partially restore impaired neuronal functions in patients with MECP2 deficiency. Clinical trial results have shown that its administration produces significant improvements in some symptoms of the disease, such as non-verbal communication, social interaction, breathing pattern, sleep, and reduction of stereotyped movements [10]. However, its use is frequently associated with side effects, particularly diarrhea, decreased appetite, vomiting, and weight loss, which may require dose adjustment or discontinuation of treatment in severe cases [11].

Although no information has been reported that allows a precise elucidation of the mechanism of action of Trofinetide, available preclinical and clinical studies suggest that it modulates different convergent processes in RTT. It is known that the administration of the drug promotes the reduction of neuroinflammation, the activation of microglia, the formation of new neuronal connections and the restoration of dendritic structure [12,13]. These effects could be related to the signaling pathways regulated by IGF-1, although a specific molecular target that explains its action has not been identified [10].

The present study aims to explore, using structure-based computational tools, the potential interactions of Trofinetide with a set of proteins relevant to the pathophysiology of Rett syndrome, to generate mechanistic hypotheses about its multitarget mode of action. A systematic approach is proposed that analyzes the coupling of Trofinetide with proteins classified into four functional groups: i) Neurotransmission, ii) Cellular metabolism, iii) Neuroinflammation and oxidative stress (OS) and iv) Drug metabolism.

Each system will be analyzed by comparison with reference ligands (endogenous substrates or recognized modulators) as functional comparators. Affinity, steric complementarity, and binding site similarity metrics will be used to assess the plausibility of a relevant direct interaction, as well as to validate the specificity of the computational protocol. This approach will allow for the validation of the appropriateness of the docking protocol, the identification of targets with potential functional relevance, and the formulation of new hypotheses regarding the molecular mechanism of action of Trofinetide, in order to guide future experimental validations and computational drug design for RTT disease.

Methodology

Target election

A combination of database searching, target prediction, and literature review was used to select potential therapeutic receptors and estimate the pharmacological efficiency of the designed compounds against RTT.

DrugBank and ChEMBL databases [25,26] were used to search for information about the Trofinetide mechanism of action and its experimental reported or computationally predicted molecular targets .

For the prediction of potential molecular targets of Trofinetide and GPE, three complementary platforms were used: SwissTargetPrediction (STP) [27], PharmMapper (PM) [28] and PASS Online (PO) [29], each with different approaches based on structural chemistry or biological activity. The selection of relevant targets was based on two main criteria: high probability or predicted affinity (Probability ≥ 0.1 in STP, NormFit > 0.8 in PM, or Pa > 0.7 in PO) and relevance in the context of Rett syndrome. This approach allows to prioritize highest biological plausibility targets.

In addition, information on phase 3 clinical trials of drugs targeting Rett syndrome was obtained through the US National Institutes of Health's ClinicalTrials.gov platform [30]. The goal is to identify therapeutic targets modulated by investigation agents with known mechanisms of action.

And at the same time, a review of the recent literature was carried out to complement the receptors with a therapeutic potential with promising potential for the condition [1-19,20-26,27,31-79]. Finally, a refinement was carried out, choosing only the receptors whose structure is reported in the protein data bank [80], to carry out molecular docking studies.

Protonation States

The deprotonation pathway of Trofinetide and GPE were estimated using the Marvin Suite platform [81] as a reference. Then, the pKa values were refined through a parametric fitting protocol based on density functional theory (DFT) electronic structure calculations [82]. The calculations were performed with Gaussian 09 [83] with a M05/6-311+G(d,p) level of theory. The accurate determination of pKa is essential in the context of drug design, since it regulates the fraction of neutral species present at physiological pH, which have a higher probability of crossing biological barriers by passive diffusion. This methodology has been previously validated, showing a satisfactory correlation with experimental values reported in the literature [84].

Molecular Docking

The structures of the crystallized proteins were obtained from protein data bank [85], the data of the proteins code and co-crystallized ligands are in the Table SX2 in supporting information (SI). Misplaced loop regions in the proteins were fixed using Modeller [86].

Water molecules, ions and non-relevant species of the protein were removed using Autodock Tools [87]. The protonation state of the side chains was considered at physiological pH= 7.4. Aspartic and glutamic acid were considered as deprotonated species. Arginine, lysine and histidine were considered with its protonated lateral chains. GROMOS96 [88] force field in the Deep View/Swiss PDB Viewer 4.1.0 software [89] were applied for protein energy minimization. Atomic charges of all ligands were estimated as single points, using the NBO protocol with the DFT theory at M05-2X/6-311+G(d,p) level. The charges of the protein were added as Kollman type [90]. All the docking simulations were carried out with AutoDock Vina 1.2.0 [91]. A gradient optimization algorithm was performed on the active or allosteric sites in the proteins (see Table SX1 in SI). For the copper-zinc superoxide dismutase (SOD) a blind docking was done since a no ligand co-crystallized structure was found in the database, then, a new docking simulation was performed in the site of major affinity. Docking scores (ΔG_B) were reported for the best pose and weighted ($\Delta G_B{}^W$) according to the molar fractions of most relevant species at pH = 7.4.

$$\Delta G_B^W = \sum_{i=1}^{n} X_{(i)} \Delta G_{B(i)}$$

Validation of the docking protocol was carried out using redocking simulations. These simulations were performed with co-crystallized ligands that, in most cases, act as reference drugs (RD). The obtained RMSD values are in the range of 0.62 to 2.99 Å. For analysis purposes, the simulations of endogenous substrates (ES) with the corresponding receptors were also performed. To compare A polygenic index (PS) was designed. Two variants were calculated: one relative to reference modulators (P_S^{RM}) and another relative to endogenous substrates (P_S^{ES}). Mathematically, these indices have the following form:

$$P_S^{RM} = log \frac{\Delta G^W{}_{B_p}}{\Delta G_{B_{RM}}} \qquad P_S^{ES} = log \frac{\Delta G^W{}_{B_p}}{\Delta G_{B_{ES}}}$$

where $\Delta G^W{}_{B_p}$ is the weighted docking score for Trofinetide or GPE and, $\Delta G_{B_{RM}}$ and $\Delta G_{B_{ES}}$ are the scores for the corresponding reference modulator and endogenous substrate respectively.

The protein-ligand conformations with the major were drawn and analyzed with Discovery Studio software [92]. Then, the interaction similarity score S_{SI} [93] was estimated to taken into account the conformation profile in relation to the endogenous substrate and modulator reference conformations. This S_{SI} is defined as follows:

$$S_{SI} = \frac{N_{MR} + N_{MI}}{2N_{RR}}$$

where N_{MR} is the number of residues present in both the compound and the reference ligand, N_{MI} is the number of exact matches in the type of interaction (hydrogen bonding, π forces, etc.) between those residues, and N_{RR} is the total number of residues involved in the interaction between the enzyme and the reference ligand.

Results

Target selection

The differences in the molecular interaction profiles between Trofinetide and GPE, observed in the results predicted by target fishing, may be partially explained by their structural differences. Although both share the GPE (Gly-Pro-Glu) core, trofinetide features a structural modification with an additional methyl group at the glutamic terminus, which improves its metabolic stability and permeability in the CNS (ref). Additionally, this modification could allow greater versatility in molecular recognition, facilitating interaction with targets such as PPARγ, MAO-B, or PTP1B, which were predicted by the platforms exclusively for trofinetide and not for GPE.

Trofinetide showed greater affinity for experimentally supported targets in RTT models, supported by the cross-reference between the targets predicted by target fishing (Table S1) and the curated data of relevant targets in RTT (Table 1). Notably, Trofinetide presented matches with GSK3β, PTP1B, MAO-B, σ_1R, and COMT, all with preclinical or clinical evidence in RTT, whereas GPE only shared AMPA and P2X7, with only indirect support for the pathophysiology of the disease. This greater concordance suggests that the structural modification of Trofinetide not only improves its pharmacokinetic profile but potentially optimizes its ability to modulate functional targets in RTT, which could contribute to its observed clinical efficacy.

In the databases reviewed and in the FDA clinical information, Trofinetide was identified as a substrate for UGT2B15 and the hepatic transporter OATP1B1, which are involved in processes related to drug metabolism and excretion, respectively. However, no interactions with cytochrome P450 enzymes were observed, suggesting a distinct elimination pathway from many neuroactive drugs. Although these proteins are not directly involved in the pathophysiology of RTT, they will be incorporated as validation controls in the molecular docking protocol: UGT2B15 and OATP1B1 as positive controls, as they have documented interactions, and CYP450 as a negative control, as it lacks them.

Finally, to define the most relevant molecular targets for docking studies, we conducted a literature review based on experimental and clinical data associated with Rett syndrome (RTT). This process included a systematic review of clinical trials, mechanistic studies, pharmacological interventions, and receptor alterations reported in RTT models. The list was refined through cross-validation with databases and expanded by incorporating targets predicted by computational methods. As a result, a total of 28 molecular targets were

selected, covering neurotransmission, synaptic plasticity, metabolic regulation, oxidative stress, inflammation, and drug metabolism. In this context, Table 1 summarizes the proteins selected for molecular docking with trofinetide, highlighting their acronym and their reported or proposed relevance in the pathophysiology of RTT, based on experimental models or clinical evidence.

Table 1. Molecular targets ans their relevance in RTT

Protein		Acronym	Relevance in RTT
Neurotransmission	N-Methyl-D-Aspartate receptor	NMDA	Its modulation improves respiratory patterns and short-term plasticity. In vivo studies with memantine, ketamine, and dextromethorphan [14,15].
	GABA transporter 1	GAT1	Its dysregulation has been observed in RTT models, affecting the excitatory-inhibitory balance [16,17].
	Metabotropic GABA receptor type B	GABAB	Altered expression has been associated with motor and behavioral dysfunction in RTT. Preclinical evidence in KO mice [14,16].
	Ionotropic GABA receptor type A	GABAA	Its imbalance with glutamatergic receptors contributes to the clinical phenotype of RTT. Targeted in GABA-based therapies [15,17].
	Muscarinic acetylcholine receptor M1	CHRM1	Cholinergic alterations have been identified in RTT models [16].
	Dopamine receptor D2	D2Dr	The D2 agonist sarizotan was evaluated in clinical trials for respiratory symptoms in RTT [15].
	Serotonin receptor 2A	5HT2A	Serotonergic dysregulation has been observed in RTT [15].
	Adenosine receptor A2A	A2AR	Activation exerts neuroprotective effects. Functional studies in neurological models suggest involvement in RTT [16].
	$Na^+/K^+/Cl^-$ cotransporter 1	NKCC1	GABA signaling analyses in RTT models show that altered function contributes to excitatory/inhibitory imbalance [16].
	Serotonin transporter	hSERT	Selective serotonin reuptake ihibitors increases MeCP2 expression and have shown partial clinical benefit in RTT [14].
	Acetylcholinesterase	AChE	Cholinergic studies in RTT show functionally relevant alterations [16].

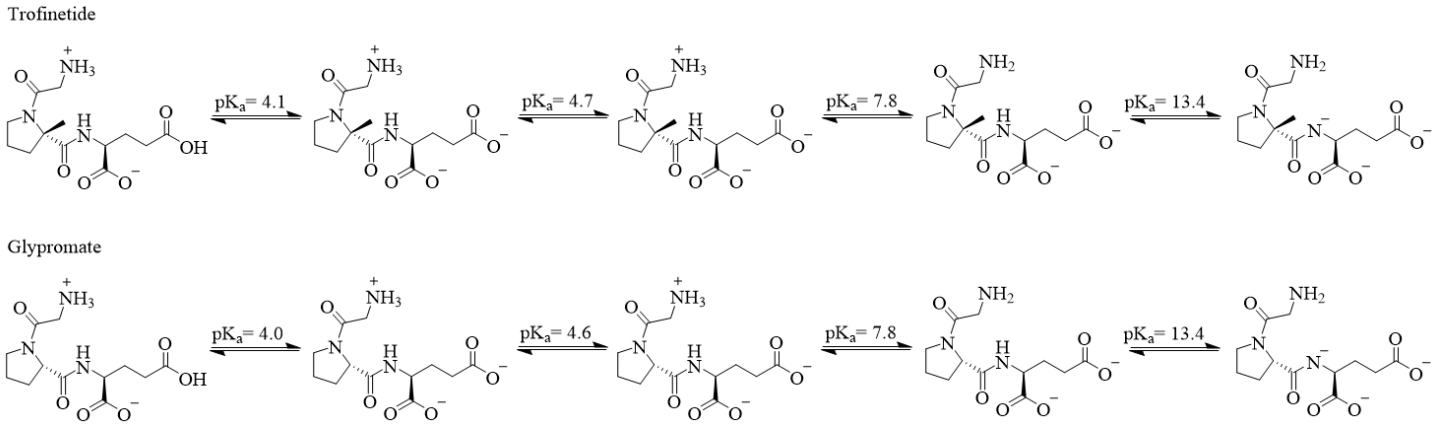
	Monoamine oxidase B	MAOB	Monoaminergic metabolism abnormalities have been described in RTT patients and models [18].
	Catechol-O-methyl transferase	COMT	May modulate dopamine and norepinephrine levels [16].
	Ionotropic AMPA-type glutamate receptor	AMPA	Glutamatergic neurotransmission is dysregulated in RTT models [16,17] .
	Sigma 1 receptor	σ_1R	Involved in neuroprotection. Preclinical evidence in RTT models. Blarcamesine an agonist is now in clinical trials against RTT [17,19].
Metabolism	Glycogen synthase kinase 3 beta	GSK3β	Implicated in neuroinflammation; proposed as a therapeutic target in preclinical RTT models [16].
	Peroxisome proliferator-activated receptor gamma	PPARγ	PPARγ agonists have shown neuroprotective effects in RTT models [20].
	Proline dehydrogenase	ProdH	Overexpressed in Mecp2-KO brains. Contributes to redox imbalance and mitochondrial damage in RTT [17].
	3-hydroxy-3-methylglutaryl-CoA reductase	HMGCR	Modulates cholesterol synthesis; inhibition improves motor symptoms and longevity in Mecp2 models [21].
	Protein tyrosine phosphatase 1B	PTP1B	Overexpressed in RTT patient brains and Mecp2-/y models. Inhibition restores PI3K–Akt signaling involved in cell survival [21].
Oxidative stress and neuroinflamation	Xanthine oxidase	XO	Implicated in systemic oxidative stress observed in RTT patients and models [17].
	Superoxide dismutase	SOD	It is reduced brains and peripheral tissues of Mecp2 models, contributing to oxidative damage vulnerability [16].
	Cyclooxygenase 2	COX-2	Participates in inflammatory processes; overexpressed in models with glial activation and oxidative damage [23].
	P2X purinoceptor 7	P2X7	Involved in microglial activation and neurotoxicity in RTT [24].
	Neuronal nitric oxide synthase	nNOS	Dysregulation may contribute to oxidative damage in Mecp2-/y brains [16].
Drug metabolism	UDP glucuronosyltransferase 2B15	UGT2B15	Identified as the main metabolic pathway for Trofinetide in clinical and preclinical studies [13].

	Organic anion transporting polypeptide 1B1	OATP1B1	Hepatic transporter involved in Trofinetide uptake; systemic role confirmed in clinical drug–drug interaction studies [13].
	Cytochrome P450 enzymes	P450	Trofinetide does not significantly interact with major CYP isoforms. Used as a methodological control [10,13].

Acid-Base equilibria

No experimental pKa values reported for Trofinetide or GPE were found in the literature, so they were determined using validated computational estimates. In the Scheme 2 the deprotonation route for both compounds is found.

Acid-base speciation analysis showed that Trofinetide and GPE exhibit virtually identical deprotonation pathways, with the successive formation of five species. At physiological pH (pH = 7.4), both molecules exhibit a distribution dominated by anionic ($X_{(H_2X)^-}$ = 0.71) and dianionic ($X_{(HX)^{2-}}$ = 0.28) species, while neutral, cationic, and trianionic forms are virtually nonexistent under these conditions. This similarity suggests that the functional differences between the two compounds are not related to their acid–base profile, but to other structural and electronic factors.



Scheme 2. Deprotonation routes for Trofinetide and GPE respectively.

Molecular docking

Affinity values weighted by the mole fraction of the predominant acid-base species at physiological pH allowed us to compare the interaction profile of Trofinetide and glypromate with a panel of 28 receptors selected for their potential relevance in Rett

syndrome. Overall, Trofinetide showed higher affinities than GPE. Although individual differences are not markedly large, overall affinities slightly favor Trofinetide.

Comparing the values of both peptides with the corresponding endogenous substrates or reference modulators included in the study revealed consistent patterns. Across multiple targets, the peptides showed higher affinity than the physiological substrates, suggesting a potential competitive modulatory capacity and consistent with the proposed multitarget activity of Trofinetide. However, the reference modulators exhibited, as expected, the highest affinities in most cases.

Regarding the validation targets used in the methodological protocol, P450, OATP1B1, and UGT2B15, the latter two have been identified as elimination pathways in clinical and preclinical studies and were therefore included as positive controls. Indeed, Trofinetide showed similar or even higher scores than the references (-8.42 and -8.08 kcal/mol, respectively), consistent with the reported pharmacokinetic data. In contrast, at P450, both peptides presented significantly lower affinities (-6.29 and -6.31 kcal/mol) than those of the endogenous substrate and the reference modulator, corroborating the absence of experimental interaction with this enzymatic system and thus validating its inclusion as a negative control.

Table 2. Weighted docking score (ΔG^{W}_{B}) of Trofinetide, Glipromate, endogenous substrates (ES), and reference modulators (MR) on selected RTT-related targets.

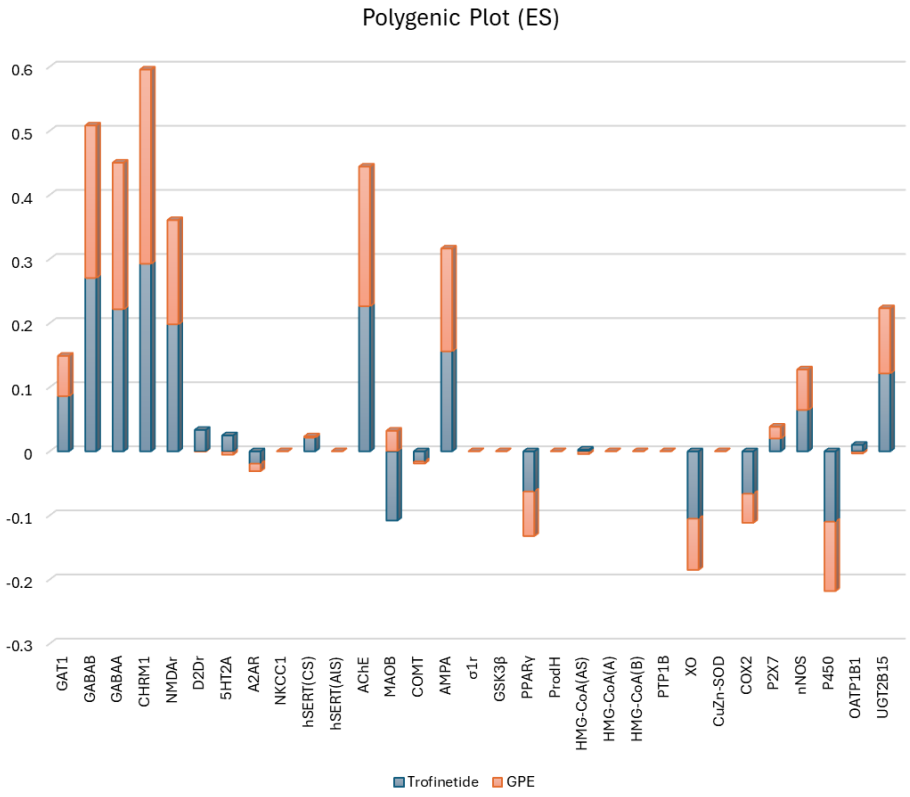
Receptor	ΔG^{W}_{B}(kcal/mol)			
	Trofinetide	GPE	ES	MR
NMDAr	-6.12	-5.80	-5.02	-6.10
GAT1	-7.64	-7.09	-4.1	-8.06
GABAB	-8.46	-8.60	-5.08	-8.43
GABAA	-6.53	-6.69	-3.33	-8.61
CHRM1	-7.50	-6.91	-4.75	-8.3
D2Dr	-6.74	-6.24	-6.243	-10.11
5HT2A	-7.24	-6.76	-6.84	-11.68
A2AR	-6.73	-6.84	-7.03	-10.23
NKCC1	-7.23	-6.96	---	-8.4
hSERT(CS)	-7.13	-6.82	-6.79	-8.89
hSERT(AlS)	-6.38	-6.11	---	-7.72

AChE	-8.25	-8.09	-4.90	-12.01
MAOB	-4.68	-6.46	-6.00	-10.00
COMT	-5.21	-5.36	-5.40	-7.60
AMPA	-6.83	-6.9	-4.77	-9.16
σ_1r	-7.92	-7.75	---	-7.88
GSK3β	-8.80	-9.36	---	-7.81
PPARγ	-6.11	-6.01	-7.05	-8.11
ProdH	2.81	1.42	-5.86	-5.72
HMG-CoA(AS)	-5.44	-5.36	-5.41	-5.77
HMG-CoA(A)	-6.27	-6.25	---	-7.19
HMG-CoA(B)	-6.31	-6.26	---	-7.83
PTP1B	-5.04	-5.00	---	-6.37
XO	-5.05	-5.35	-6.43	-5.65
CuZn-SOD	-6.78	-7.35	---	-6.94
COX2	-6.58	-6.90	-7.66	-8.80
P2X7	-8.95	-8.92	-8.55	-7.49
nNOS	-7.26	-7.24	-6.26	-7.13
P450	-6.29	-6.31	-8.096	-9.65
OATP1B1	-8.42	-8.17	-8.229	-8.20
UGT2B15	-8.08	-7.72	-6.109	-6.42

To more precisely compare the relative affinities of Trofinetide and GPE for endogenous substrates (ES) and reference modulators (RM), the polygenic score was estimated. A positive value indicates that the compound has a higher affinity than the reference substrate or modulator, while a negative value indicates the opposite.

The graph based on endogenous substrates (Figure 1, top) revealed that both trofinetide and GPE displayed higher affinities for several targets involved in neurotransmission, including GABAB, GABAA, GAT1, and CHRM1, among others. Overall, Trofinetide displayed slightly higher scores than GPE for this set of targets, suggesting a more favorable interaction profile. In contrast, GPE displayed a higher score only for MAO-B, which could reflect differential modulation of this signaling pathway. Notably, both compounds exhibited positive values for P2X7, a receptor with experimental implications for microglial activation in models of Rett syndrome (ref). In contrast, the reference modulator-based

graph (Figure 1, bottom) showed predominantly negative values, which was expected since these compounds were specifically designed to exhibit high affinity for their respective receptors. However, Trofinetide maintained a competitive relative affinity against several pathophysiologically relevant targets, such as GSK3β, nNOS, and P2X7, all of which are linked to neuroinflammation and oxidative stress processes in RTT. Once again, these findings reinforce the hypothesis of a possible multitarget modulatory effect of Trofinetide.



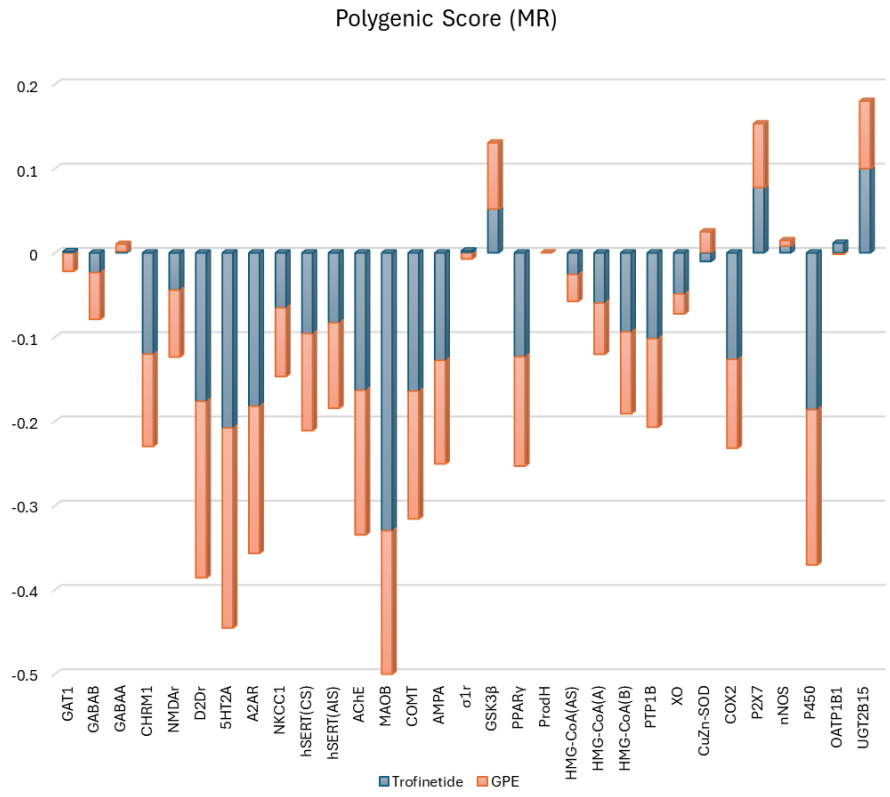


Figure 1. Trofinetide and GPE polygenic score plot relative to endogenous substrate (top) and reference modulator (bottom).

Conclusions

Acknowledgements

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