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LAT-1 activity of *meta*-substituted phenylalanine and tyrosine analogs

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ABSTRACT

The transporter protein Large-neutral Amino Acid Transporter 1 (LAT-1, SLC7A5) is responsible for transporting amino acids such as tyrosine and phenylalanine as well as thyroid hormones, and it has been exploited as a drug delivery mechanism. Recently its role in cancer has become increasingly appreciated, as it has been found to be up-regulated in many different tumor types, and its expression levels have been correlated with prognosis. Substitution at the *meta* position of aromatic amino acids has been reported to increase affinity for LAT-1; however, the SAR for this position has not previously been explored. Guided by newly refined computational models of the binding site, we hypothesized that groups capable of filling a hydrophobic pocket would increase binding to LAT-1, resulting in improved substrates relative to parent amino acid. Tyrosine and phenylalanine analogs substituted at the *meta* position with halogens, alkyl and aryl groups were synthesized and tested in *cis*-inhibition and *trans*-stimulation cell assays to determine activity. Contrary to our initial hypothesis we found that lipophilicity was correlated with diminished substrate activity and increased inhibition of the transporter. The synthesis and SAR of *meta*-substituted phenylalanine and tyrosine analogs is described.

The Large-neutral Amino Acid Transporter 1 (LAT-1, SLC7A5) has received considerable attention as both a drug delivery mechanism to access the BBB;[1–5] and for its role in cancer, where it is up-regulated in many different types of human tumor cells, including prostate,[6] colorectal,[7] non-small-cell lung cancer (NSCLC)[8] and glioblastoma multiforme (GBM).[9,10] Furthermore LAT-1 expression has been correlated with poor prognosis for cancer patients,[11–14] and tumor growth can be repressed by use of LAT-1 inhibitors[15–19] presumably due to a cancer cell's increased nutritional demands.

A challenge for using LAT-1 in drug delivery is the problem of transporter saturation by endogenous amino acids, which have plasma concentrations significantly higher than their Michaelis constant (K_m) value.[20] One approach to solving this problem would be to develop drugs or prodrugs with increased affinity (or reduced K_m values) for LAT-1 in comparison to the K_m values of natural amino acid substrates (K_m values of 11–210 μM),[20] while at the same time not sacrificing maximal velocity V_{max}. It has been shown that the substitution pattern for aromatic amino acids can have a dramatic effect on LAT-1 activity, with *meta* substitution significantly improving binding affinity relative to other positions.[21,22] And though transporter substrates are desirable for drug delivery applications, potent LAT-1 inhibitors could also be useful as metabolic blockers in cancer. To date, the LAT-1 SAR for substituted aromatic amino acids has only received a cursory exploration, with significant gaps remaining to be filled. As part of our objective to identify improved transporter substrates, we sought to expand the current understanding of the effect of substitution at the *meta* position for a series of phenylalanine and tyrosine analogs.

The atomic structure of the human LAT-1 is not known; however, we have developed a homology model of LAT-1 based on a structure of a related transporter, the arginine–agmatine transporter AdiC from *Escherichia coli*.[23] The model can rationalize amino acid selectivity among amino acid transporters, and virtual screening against this model followed by experimental testing identified previously unknown LAT-1 ligands.[24] Based on our previously published LAT-1 model,[23] which was subsequently refined (Supplementary material), as well as those models developed by others[25,26] we hypothesized that *meta* substitution on the aromatic ring of phenylalanine and tyrosine with lipophilic groups capable of filling a hydrophobic pocket (**PA**, Fig. 3) in the LAT-1 binding site

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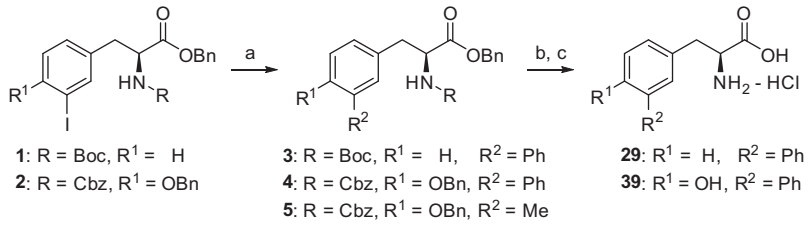
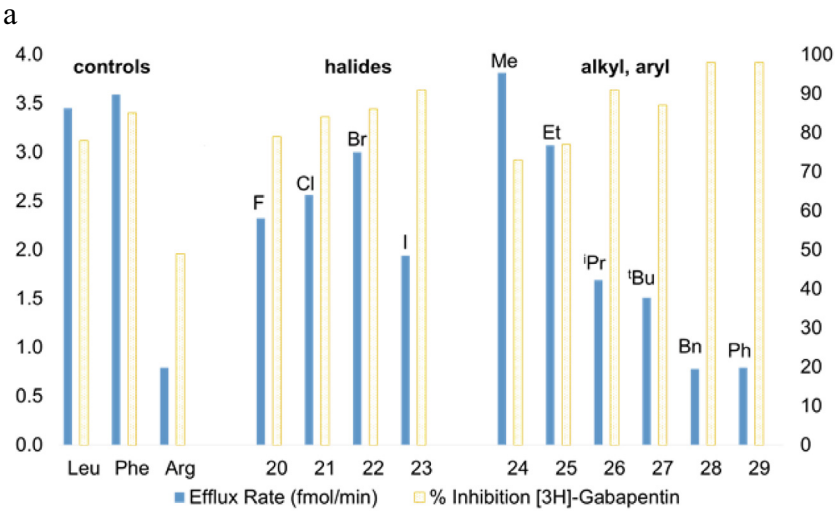
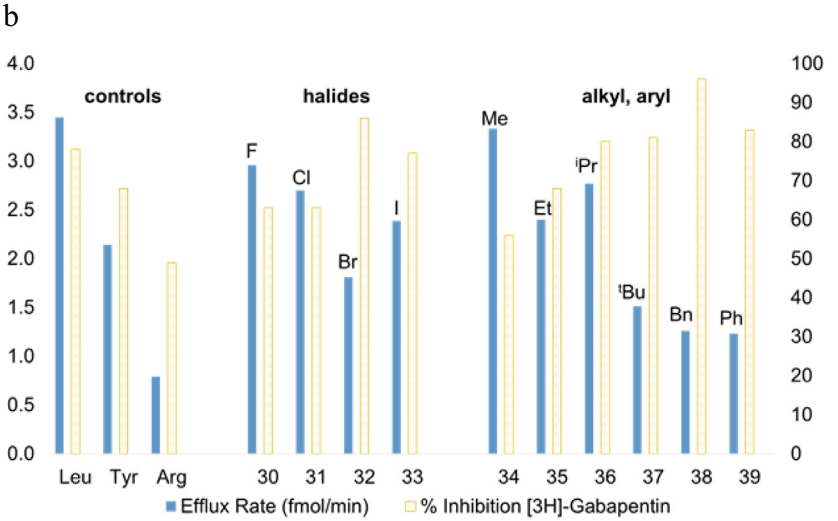


1: R = Boc, R^1 = H
2: R = Cbz, R^1 = OBn
3: R = Boc, R^1 = H, R^2 = Ph
4: R = Cbz, R^1 = OBn, R^2 = Ph
5: R = Cbz, R^1 = OBn, R^2 = Me
29: R^1 = H, R^2 = Ph
39: R^1 = OH, R^2 = Ph

Scheme 1. Synthesis of compounds **29** and **39**. Reagents and conditions: (a) $PhB(OH)_2$, Na_2CO_3, $Pd(OAc)_2$, P(*o*-tolyl)$_3$, DME/H_2O (6:1), 80 °C, **3**: 63%, **4**: 52%, **5**: not isolated; (b) H_2 (balloon), Pd/C; (c) 1:1 6 N HCl (aq)/1,4-dioxane, **29**: 35%, **39**: 25% (prep. HPLC purification).





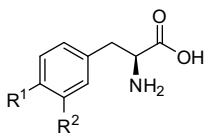


Figure 1. The effect of *meta* substituents (R^2) on exchange efflux rate and uptake of [^{3}H]-gabapentin in HEK-hLAT1 cells for (a) L-phenylalanine (R^1 = H) and (b) L-tyrosine (R^1 = OH) analogs. Data represents the mean of at least three experiments. Positive controls L-leucine, L-phenylalanine, and L-tyrosine are known LAT-1 substrates; and negative control L-arginine is not.[20] The left ordinate indicates efflux rate (fmol/min) in which compounds were tested at 200 μM for their ability to cause efflux of [^{3}H]-gabapentin from preloaded HEK-hLAT1 cells. Average SD ± 0.2 fmol/min. The right ordinate indicates % inhibition in which compounds were tested at 200 μM for their ability to inhibit uptake of [^{3}H]-gabapentin into HEK-hLAT1 cells. Data is presented as % inhibition relative to background signal in the absence of an inhibitor. Average SD ± 2%.

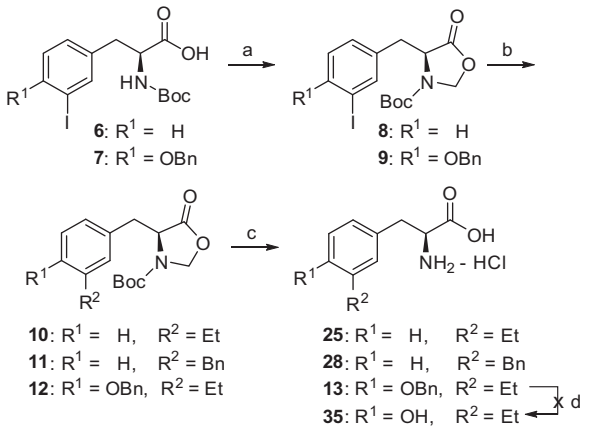
might lead to an increase in LAT-1 binding and an improvement in substrate activity.

To evaluate substrate activity, compounds were tested in two different cell-based assays, using a LAT-1 overexpressing cell line generated by transfection of HEK cells with human LAT-1 cDNA.[24] HEK-LAT1 cells were used to identify ligands for LAT-1 using both *cis*-inhibition and *trans*-stimulation assays.[24] *cis*-Inhibition involves competition of test compound with a radiolabeled amino acid substrate (e.g., [^{3}H]-gabapentin) for LAT-1 mediated cellular uptake. Though the *cis*-inhibition assay can identify agents which interact with LAT-1, it is exclusively used to identify inhibitors, not substrates of the transporter. To determine whether a compound was a substrate, a *trans*-stimulation assay[27] was performed. This assay exploits LAT-1's alternating access mechanism[28,29] by pre-loading cells with [^{3}H]-gabapentin followed by incubation with test compound. We choose [^{3}H]-gabapentin as a probe substrate due to its selectivity against other transporters.[29] The efflux rate of radiolabeled amino acid in the presence of the test compound is compared to the efflux rate in the absence of the test compound and with both positive and negative control amino acids (leucine and arginine, respectively) and parent amino acids (phenylalanine and tyrosine) to assess whether a test compound is a LAT-1 substrate. Substrates enhance the efflux rate of the radiolabeled amino acid compared with its efflux rate in the absence of the substrate.

To prepare compounds **29** and **39**, protected L-phenylalanine **1** and L-tyrosine **2** were subjected to a Suzuki coupling[30] with phenylboronic acid followed by deprotection according to Scheme 1. Attempts to introduce a methyl group at R^2 using Suzuki coupling with methyl boronic acid were unsuccessful,[31] and NMR analysis of the reaction mixture for conversion of **2** to **5** indicated low yield (<25%) of desired product (not isolated).

In order to prepare the desired alkyl-substituted amino acids of Figure 1, we decided to examine the Negishi coupling[32] as an alternative to the Suzuki reaction. Initial attempts to perform Negishi coupling with diethyl zinc as a test case on **1** or **2** using conditions described for a protected amino acid[33] gave poor yields (<20%) of the desired 3-ethyl substituted phenylalanine or tyrosine derivative. A literature search revealed no examples of Negishi coupling reactions between iodo-substituted phenylalanine or tyrosine analogs (e.g., **1** or **2**) and alkyl zinc reagents. Apparently, Negishi coupling of aromatic amino acids has been limited to substitution with cyano[34] or aromatic rings.[35] We hypothesized that the desired Negishi coupling might give a better yield if both the carbamate 'N–H' and carboxylic acid of **6** and **7** were masked, and this prompted us to explore an oxazolidinone[36,37] protecting group (Scheme 2). To our knowledge, this synthetic method is a new approach for obtaining *meta*-substituted phenylalanine derivatives. In addition to serving the dual purpose of protecting both the carbamate and carboxylic acid, the oxazolidinone was easily removed under acidic conditions to simultaneously remove the Boc group giving the *meta* substituted amino acids **13**, **25** and **28**. Unfortunately the desired ethyl substituted tyrosine **35** was not obtained using this route due to failure to deprotect the benzyl ether of **13** presumably caused by poisoning of the palladium catalyst used in hydrogenolysis.[38]

As deprotection of tyrosine intermediate **13** (Scheme 2) was problematic, and moreover, we desired bulky alkyl groups at R^2 (i.e., isopropyl and *tert*-butyl) that could be problematic using the cross coupling strategy of Scheme 2 (e.g., conversion of **8** to **10**), we explored the convergent approach depicted in Scheme 3 as an alternative. Conversion of commercially available alkyl iodide **14** to Jackson's organozinc **15**[39,40] followed by an in situ Negishi coupling[41] to give **17a–f** was successfully performed. Though this route added additional synthetic steps to obtain noncommercial *meta* substituted aryl iodides[42] **16c–f**, we found Scheme 3 to be a



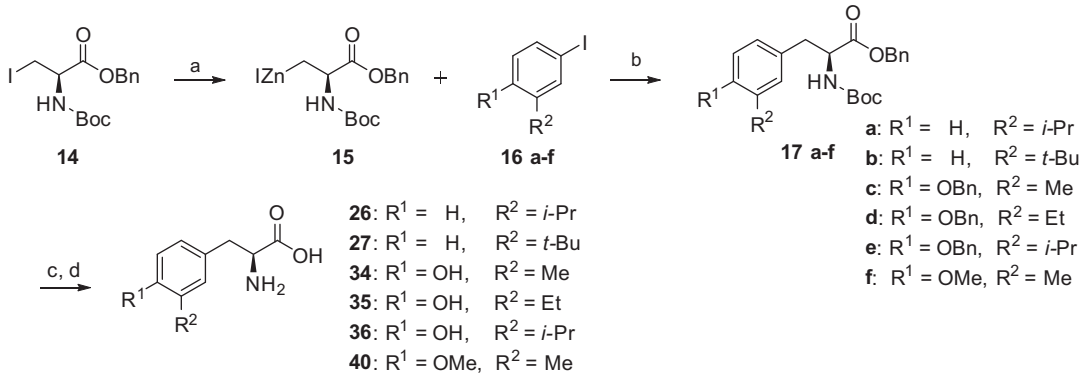
Scheme 2. Synthesis of compounds **25** and **28**. Reagents and conditions: (a) K_2CO_3, CH_2Br_2, CH_3CN, 100 °C, **8**: 43%, **9**: 32%; (b) Et_2Zn, $PdCl_2$(dppf)–DCM, THF, rt, 18 h, **10**: 74%, **11**: 60%, **12**: 62%; (c) 1:1 6 N HCl (aq)/1,4-dioxane, 60 °C, **25**: 60%, **28**: 38%, **13**: 29%; (d) H_2 (balloon), Pd/C, no reaction.

robust approach for preparing several of the desired amino acid analogs of Figure 1 (i.e., **26**, **27**, **34–36**, and **40**).

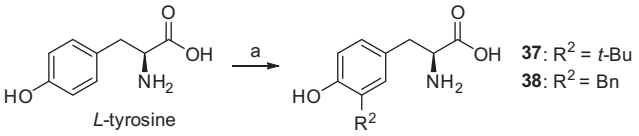
Though tyrosine analogs **37** and **38** (R^2 = *tert*-butyl and benzyl, respectively) could potentially be synthesized using the approach of Scheme 3, we employed a different method[43] for these two compounds, taking advantage of the reactivity of tyrosine toward electrophilic aromatic substitution. Use of *tert*-butyl alcohol as a source of *tert*-butyl cation gave almost exclusively regioisomer **37**, whereas, the analogous reaction with benzyl alcohol gave a mixture of products, presumably resulting from di- and tri-benzylation.[44,45] Fortunately, the desired product **38** was easily separated from the other isomers by preparative HPLC.

Tyrosine and phenylalanine analogs synthesized in Schemes 1–4, were evaluated in *cis*-inhibition and *trans*-stimulation assays (Fig. 1).[24] Halogen size for phenylalanine derivatives **20–22** correlated with an increase in substrate activity (blue bars, Fig. 1), but this trend failed to hold at iodide **23**, as a significant decrease was observed (Fig. 1a). In contrast, a consistent trend of increasing % inhibition (yellow bars, Fig. 1) with size was observed for the halogens. A more noticeable trend in activity was observed for alkyl- and aryl-substituted phenylalanines (**24–29**). Though methyl (**24**) was found to be slightly better than hydrogen (i.e., parent Phe), larger, more lipophilic groups (benzyl and phenyl in **28** and **29**, respectively) lead to loss of substrate activity, but a rise in % inhibition. *c*Log*P* values[46] indicate that the lipophilicity of iodo (**23**: −0.43) lies in between an ethyl (**25**: −0.53) and isopropyl group (**26**: −0.13). This order roughly matches what is observed with activity. For comparison sake, compounds **28** and **29** have substantially higher *c*Log*P* values (+0.51 and +0.33, respectively), and they both exhibited 98% inhibition of [^{3}H]-gabapentin in the *cis* assay. IC_{50} values for compounds **28** and **29** were determined to be 7.3 and 6.6 μM, respectively. This level of transporter inhibition is comparable to lipophilic thyroid hormones (e.g., triiodothyronine), which also demonstrated poor substrate activity that correlated with lipophilicity.[25] However, clearly other factors besides lipophilicity impact activity as methyl analog **24**'s *c*Log*P* value (−1.1) lies in between fluoro (**20**: −1.4) and chloro (**21**: −0.84), yet **24** had the greatest substrate activity of all the tested compounds (efflux rate = 3.8 fmol/min).

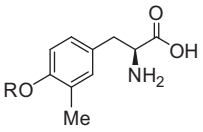
Surprisingly, a different trend was apparent for halogenated tyrosines (**30–33**, Fig. 1b), where the best substrate contained a *meta* fluoro substituent (**30**). And there was a decrease in activity going from chloro (**31**) to bromo (**32**), with a slight, but significant increase in substrate activity from bromo (**32**) to iodo (**33**)



Scheme 3. Synthesis of compounds **26**, **27**, **34**–**36**, and **40**. Reagents and conditions: (a) Zn, 1,2-dibromoethane, TMSCl, DMF, 60 °C for 30 min to activate zinc, then sonication with **14** for 30 min; (b) Pd_2dba_3, P(*o*-tolyl)$_3$, DMF, rt, 42–57%; (c) H_2 (balloon), $Pd(OH)_2$/C, rt; (d) 1:1 6 N HCl (aq)/1,4-dioxane, 60 °C, 1 h, 28–91% (purification by prep. HPLC or crystallization).

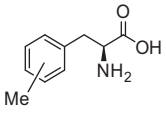


Scheme 4. Synthesis of compounds **37** and **38**. Reagents and conditions: (a) H_3PO_4, R^2OH, 65 °C, 3 d (purification by prep. HPLC).



	Efflux Rate (fmol/min)	% Inhibition at 200 μM
34: R = H	3.3	56
40: R = Me	2.9	75

Figure 2. Effect of replacing tyrosine's phenolic hydroxy with a methoxy group.



	Efflux Rate (fmol/min)	% Inhibition at 200 μM
24: *meta*	3.8	73
41: *ortho*	2.7	98
42: *para*	2.7	86

Figure 3. Comparison of methyl substituted phenylalanine isomers.

that was inversely related to % inhibition. At first glance the observed activities for the halogenated tyrosines appear difficult to rationalize; however, there is a notable correlation of substrate activity with published experimental p*K*a values for *ortho*-halophenols (F: 8.73, Cl: 8.51, Br: 8.39, and I: 8.46).[47] It is unclear to us what role, if any, tyrosine's phenolic OH might have on interaction with the transporter, and this correlation could be a coincidence. As with the phenylalanine analogs, there was a general trend toward decrease in substrate activity and rise in % inhibition as alkyl and aryl substituents became more lipophilic. One exception to the trend was isopropyl-substituted tyrosine **36**, which demonstrated markedly greater substrate activity than the corresponding isopropyl phenylalanine **26**. Whether this is due to having reached some optimal balance between the overall polarity of the phenol ring and the size and/or lipophilicity of the isopropyl group (**36**: *c*Log*P* = −1.0) or some other factor is uncertain. Since tyrosine has been shown to be an inferior LAT-1 substrate to phenylalanine in both our experiments and by others,[20] presumably due to its polar phenolic hydroxyl group causing a drop in K_m, we were pleasantly surprised to see that most of the tyrosine derivatives benefited from substitution, with *meta*-methyltyrosine **34** having comparable activity to the positive control L-leucine (3.3 vs 3.5 fmol/min, respectively). Compound **38** (R^2 = benzyl), one of the poorer tyrosine substrates gave an IC_{50} value of 9.1 μM, that was comparable in potency to **28** and **29**. An attempt to mask the polarity of the phenol hydroxy by converting it to a methoxy group, while maintaining the preferred *meta* methyl substituent (**40**, Fig. 2) resulted in a slight decrease in substrate activity relative to its comparator **34**.

It was previously shown that substitution at phenylalanine's *meta* position resulted in increased LAT-1 activity and greater brain uptake relative to the para position, though the corresponding *ortho* isomer was not tested.[21,48] To verify this trend for the current series, we tested both the *ortho*- and *para*-methylphenylalanine analogs (Fig. 3: **41** and **42**, respectively). Both isomers had identical efflux rates (2.7 fmol/min), consistent with their being inferior

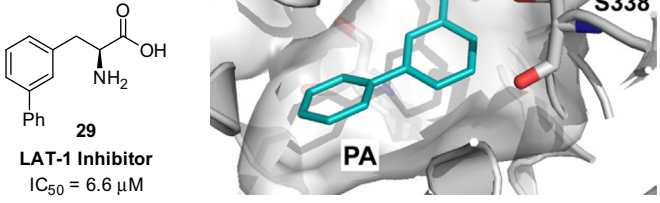


Figure 4. Predicted mode of interaction of inhibitor **29**. Docking pose of inhibitor **29** in the substrate binding site of LAT-1. Inhibitor **29** is predicted to interact with the hydrophobic subpocket **PA**, which likely contributes to the increased affinity of this compound.

substrates relative to the *meta* isomer (**24**: 3.8 fmol/min). Interestingly, the *ortho* isomer (**41**) demonstrated the greatest % inhibition (98%) of the three compounds.

We used the newly discovered substrates and inhibitors from Figure 1 to refine our models[23,24] with homology modeling and ligand docking as previously described[49] (Supplementary data). From this refined model and consideration of our SAR data, we propose that large lipophilic groups such as phenyl in **29** are causing an increase in binding affinity (lower K_i) due to hydrophobic interactions with a subpocket **PA** (Fig. 4) at the expense of decreased transport capacity (lower V_{max}) resulting in compounds being inhibitors rather than substrates. Future work will be aimed at identifying functional groups and substitution patterns on amino acid scaffolds to promote a dipole–dipole interaction with residues in the binding site (e.g., with S338) with the idea of finding an optimal balance between K_m and V_{max} in order to improve substrate activity.

We have demonstrated that *meta* substitution of tyrosine or phenylalanine with large lipophilic moieties would likely not be beneficial for designing prodrugs, as they would tend to be inhibitors rather than substrates. However, lipophilic amino acids such as **29** might be a starting point for optimization to obtain potent LAT-1 inhibitors that could be useful as cancer probes and possibly as drugs, assuming potential metabolic liabilities with universally inhibiting LAT-1 could be addressed.[15,50] Taken together the SAR trends for these *meta* substituted aromatic amino acids suggest a correlation of substituent lipophilicity with substrate activity up to some limit, and once passed this limit increasing the lipophilicity causes a reversal in activity, as compounds trend toward being inhibitors rather than substrates. Our observations are consistent with those made earlier by Uchino.[25] The definition of this lipophilicity limit likely varies in a complex manner with the properties of other ring substituents and their effect on both substrate K_m and V_{max}.

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Supplementary data

Supplementary data (full experimental details, compound characterization data, cell assay data in tabular format, IC_{50} curves, and model refinement and ligand docking description) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.04.023.

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