

# N-Myristoyltransferases as antileishmanial targets: a piggyback approach with benzoheterocyclic analogues

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Leishmaniasis is one of the neglected diseases that remain in need for pharmacological alternatives. In this context, N-Myristoyltransferases (NMT) arise as interesting targets to explore since they are involved in the co/post-translational processing of peptides which are responsible for host cell invasion. Studies that consider these enzymes as targets point out the potential of benzoheterocyclic compounds as inhibitors of *Candida albicans*'s N-myristoyltransferase. Here we applied a combination of comparative binding site analysis and molecular docking studies based on a Piggyback approach in the search for new *Leishmania major* NMT ligands. Our results revealed that NMT enzymes from both pathogens present enough structural similarity to allow extrapolation of the knowledge available from *C. albicans* studies to develop new *L. major* NMT inhibitors. Molecular docking studies with benzoheterocyclic analogues indicate the potential of benzothiazole derivatives as *L. major* NMT ligands, giving rise to a completely new class of chemical compounds to be explored in the development of antileishmanial drugs.

**Keywords:** N-myristoyltransferase. Leishmaniasis. Benzofuran. Benzothiazole. Molecular docking. *Leishmania major*.

## INTRODUCTION

N-Myristoyltransferases (NMT) are fundamental enzymes to processes of addressing protein to the plasmatic membrane, stabilizing their structures and mediating protein interactions (Alvar *et al.*, 1997; Bhatnagar *et al.*, 1999; Martin, Beauchamp, Berthiaume, 2011). They participate in the co/post-translational peptide processing by linking a myristic acid unit to the amino terminal residue of a targeted protein (Bowyer *et al.*, 2008; Brannigan *et al.*, 2010; Martin, Beauchamp, Berthiaume, 2011).

These enzymes are present in several species including humans (Duronio, Reed, Gordon, 1992), fungi (Lodge *et al.*, 1994; Towler *et al.*, 1987; Wiegand *et al.*, 1992) and parasites (Gunaratne *et al.*, 2000; Price *et al.*, 2003); are essential to the growth and survival of several eukaryotes, a fact that makes them interesting targets to explore in the search for new anti-infective therapies

(Bowyer *et al.*, 2008; Mills *et al.*, 2007; Price *et al.*, 2003; Resh, 2012; Tate *et al.*, 2014).

Leishmaniasis is a set of infectious diseases that afflicts more than 350 million people worldwide. Approximately 90% of the cases occur in developing countries such as Bangladesh, India and Brazil, a number that reflects the strong rural and neglected epidemiological profile of this disease (PAHO, 2015; WHO, 2017). Leishmaniasis's etiological agents are kinetoplastids from the genus *Leishmania* that also present NMT as one of their co/post-translational protein processing enzymes. *Leishmania (Leishmania) major*'s NMT, for instance, is an essential enzyme to the host invasion capability of this parasite and presents 41, 43, and 40% of sequence similarity with *Saccharomyces cerevisiae*, *Candida albicans* and *Plasmodium falciparum* NMT enzymes, respectively (Maurer-Stroh *et al.*, 2002).

Small molecules with inhibitory activity over these enzymes have already been reported. Benzofuran analogues were studied by Ebara *et al.*, and resulted in excellent inhibitors of *Candida albicans* NMT (CaNMT), with minimum inhibitory concentration (MIC) values

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around 0.1  $\mu\text{M}$  (Ebara *et al.*, 2005; Ebiike *et al.*, 2002; Masubuchi *et al.*, 2003). Yamazaki *et al.* have also explored benzothiazole structures because of their potential activity against CaNMT. After structural optimizations guided by systematic rational modifications, inhibitors with high affinity and selectivity were obtained (Ohtsuka, Aoki, 2003; Yamazaki *et al.*, 2005).

Evaluating the structures of both classes of reported inhibitors, at least three similar structural elements are noticeable: the presence of a benzoheterocyclic core with two main side chains, at  $C_2$  and  $C_{4/6}$  positions of the benzoheterocyclic system; a secondary amine at  $C_{4/6}$  side chain, and a hydrophobic group linked to the  $C_2$  side chain (Figure 1A).

More recent reports have shown the efficacy of benzoheterocyclic molecules in inhibiting the *Plasmodium falciparum* NMT (PfNMT) (Brannigan *et al.*, 2010; Tate *et al.*, 2014). These studies were carried out based on the Masubuchi *et al.* developments and employed a methodology known as Piggyback Approach (Gelb, 2007). This methodology saves a lot of time and money because it repurposes a well-designed work, usually found ineffective for its original design, to advance quickly in the process of a new drug development. Frearson *et al.* also identified a series of sulfonamide analogues with high affinity to the *Trypanosoma brucei* NMT (TbNMT), confirming the potential of these enzymes as suitable and

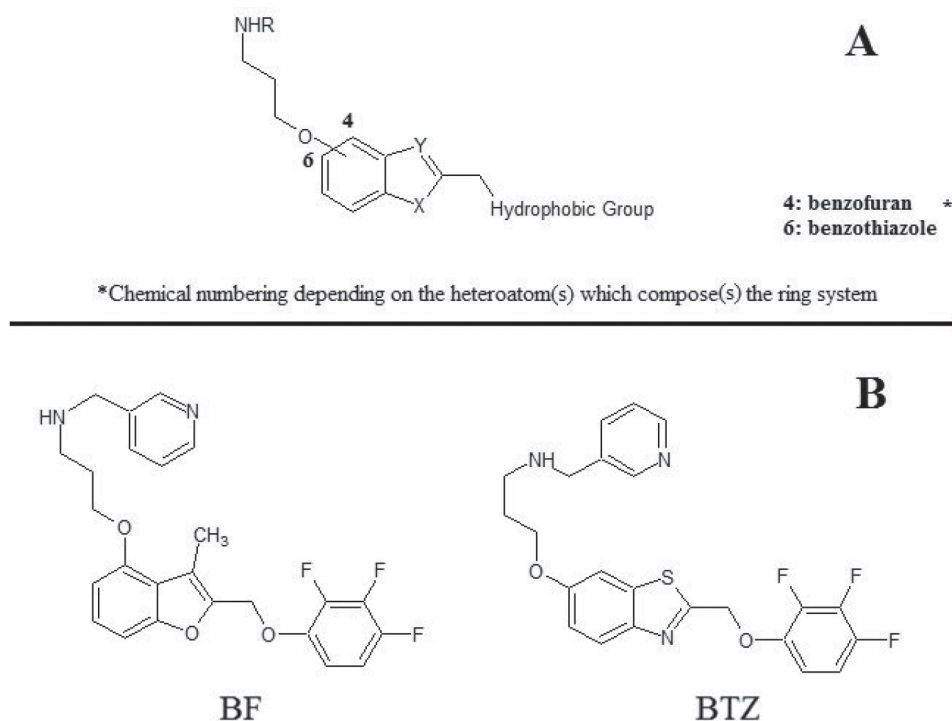
valid targets in the search for new anti-trypanosomatids (Frearson *et al.*, 2010).

In the present work, we carried out a comparative analysis between the crystallized binding sites of LmNMT and CaNMT, followed by docking studies of a benzofuran analogue (BF), which have been reported as active against the CaNMT. We also suggested the docking studies of a benzothiazole analogue (BTZ) that resembles, as closely as possible, the structural features of BF, with the rationale of observing how such characteristics modulates a possible binding mode at LmNMT (Figure 1B).

This work is also based on the principles of Piggybacking, which is an attractive approach for developing therapeutic alternatives for leishmaniasis, a neglected disease. Moreover, LmNMT is a novel and poorly explored target that could provide a whole new field in the research for antileishmanial compounds.

## MATERIAL AND METHODS

Structural comparison between the binding sites of CaNMT and LmNMT was carried out by employing crystal structures retrieved from Protein Data Bank under the entry codes 1iyl (3.2 Å resolution) and 3h5z (1.49 Å resolution) for CaNMT and LmNMT respectively, as reference. Both enzymes were preprocessed by adding missing hydrogens and by removing water molecules.



**FIGURE 1** - General structure of the reported inhibitors of CaNMT (A) and the chemical structures of the specific compounds studied herein (B).

Visual inspection for structural errors, mainly at the binding site region, was also performed, followed by a revision of the PDB files for significant structural comments, such as missing residues.

Structures were superimposed in the Maestro 10.4 molecular visualizer to perform the visual structural comparison. Connolly Molecular Surfaces were constructed employing the Molecular Surface module of this software and by setting the surface resolution to “Medium”, the probe sphere radius of 1.4 Å and a VDW radius scale of 1.0.

The similarity index was calculated by employing ProBis Web-Server (Konc, Janežič, 2010). Both PDB preprocessed structures were input at the server and the chain A was defined as the one exhibiting the binding site. “Local Alignment Only” and a Z’score value “Cutoff” of 1.0 were employed as Additional Settings to this analysis.

BF and BTZ were sketched in the GaussView 5.0.9 software and had their structural geometries optimized by first considering MM+ parameters, followed by a local minima conformer search up to energy differences of 0.01 kcal/mol and considering semi empirical methods (PM3). The single point energy calculation was then performed by employing the Gaussian 09W software, with PM3 force field.

Compounds were docked fifteen times at CaNMT and LmNMT enzymes using the Gold 5.4.0 software to generate 10 solutions each, producing a final pool of one hundred and fifty docked poses to be analyzed to each compound. A Myristoyl-CoA molecule was considered at the cofactor binding site throughout LmNMT docking studies. As reference to the binding site, it was considered a region of 14 Å around the terminal leucine residue (Leu451 at CaNMT and Leu421 at LmNMT). Moreover, a hydrogen bond interaction between the terminal leucines and the aliphatic nitrogen present in the side chain at C<sub>4</sub> or C<sub>6</sub> position of the ligands was set as constraint.

This standard protocol was established after redocking studies of CaNMT and its co-crystallized ligand, the (1-methyl-1*H*-imidazol-2-yl)-(3-methyl-4-{3-[pyridine-3-ylmethyl]-amino}-propoxy)-benzofuran-2-yl) methanone, a benzofuran analogue with chemical structure different from the BF test compound (Supplementary Information).

The redocking results were analyzed by considering two score functions, Goldscore and Chemscore, which presented the best scoring for the co-crystallized ligand, and three criteria of interaction that mimic the findings of Masubuchi et al. to benzofuran derivatives at CaNMT: a) distances between the aliphatic nitrogen of the side chain at C<sub>4</sub> or C<sub>6</sub> positions of the ligands and the carboxylic

oxygen of C-terminal leucine residues (Leu451 at CaNMT and Leu421 at LmNMT); b) distances between the heteroatom that can act as a hydrogen bond acceptor in the heterocyclic ring and the histidine from S2 region (His227 and His219 to CaNMT and LmNMT, respectively); c) orientation of the hydrophobic substituent in C<sub>2</sub> of the heterocyclic systems towards the hydrophobic region S3. Distances lower than 4Å were considered as indicative of potential molecular interactions, since the crystallographic data from Sogabe et al. showed interactions at distances up to this value (Sogabe *et al.*, 2002).

The best docked poses from docking studies were analyzed by employing the software Maestro (v.10.4), by considering the two score functions, Goldscore and Chemscore and by applying the aforementioned criteria to the redocking studies.

## RESULTS AND DISCUSSION

Superimposition of chain A from both crystals exhibited a similarity index of 4.21, represented in terms of Z-score (Probis Web-based software), a statistical function that represents how much an alignment differs from the mean obtained when considering the whole population of possible alignments. Z-score values above 2 are considered highly significant (Konc *et al.*, 2012).

Visual evaluation was also applied to compare the positioning of the proteins binding site residues, precisely in their active state. Both crystals were obtained and described as in their active conformations.

Residues involved in interactions with the benzofuran analogue co-crystallized with CaNMT from the Masubuchi *et al.* (2003) studies were considered as a reference to determine the binding site of LmNMT (Ebara *et al.*, 2005; Ebiike *et al.*, 2002; Masubuchi *et al.*, 2003). According to their findings, the binding residues are organized to produce three major pockets: S1, S2 and S3 (Figure 2A). A similar positioning of equivalent residues at LmNMT produces the same three major sites, but with some significant differences (Figure 2B).

S1 configures the catalytic region with the C-terminal leucine (Leu451 at CaNMT and Leu421 at LmNMT) playing a crucial role in the transfer of the Myristoyl-CoA subunit to the substrates. As it may be noted, this region is much larger in the CaNMT than in the LmNMT. This seems to be explained mostly by the fact that the equivalent tyrosine residues, Tyr119 and Tyr92, take a different steric positioning, with the aromatic ring of the Tyr92 occupying the space between the Leu421 and the Phe90. The same region at CaNMT is located between Leu451 and Phe117 and is not filled by any residue. Additionally, the aromatic

ring of the Tyr119 is positioned 90 degrees to the aromatic ring of Tyr107 which could indicate a potential Pi-stacking interaction with this residue.

S2 region, in turn, revealed to be the most preserved region between the two enzymes, with the histidine residues (His227/219, CaNMT/LmNMT) occupying the same position in both crystals. Masubuchi *et al.* reported this histidine as one of the main interaction points, specifically interacting with the heteroatom of the benzoheterocycle (Masubuchi *et al.*, 2003).

The main differences observed between the two crystals were, however, at S3 region. This region of CaNMT is composed by three phenylalanine residues, Phe115, Phe240, Phe339, generating a tight hydrophobic cavity. The same region at LmNMT presented a replacement of one of the phenylalanine residues (Phe339) for a serine, the Ser330, a polar amino acid that promotes, accordingly, a little increase in the polarity of this cavity at LmNMT. Moreover, this pocket was slightly larger than its equivalent at CaNMT, a fact that could also be explained by the smaller size of the Ser330 residue when compared to the Phe339.

Intriguingly, the residues Phe90 and Tyr345, while in this crystallized active conformation of LmNMT, generate a steric salience between S1 and S3 pockets that could interfere with the accommodation of more hindered or rigid ligands.

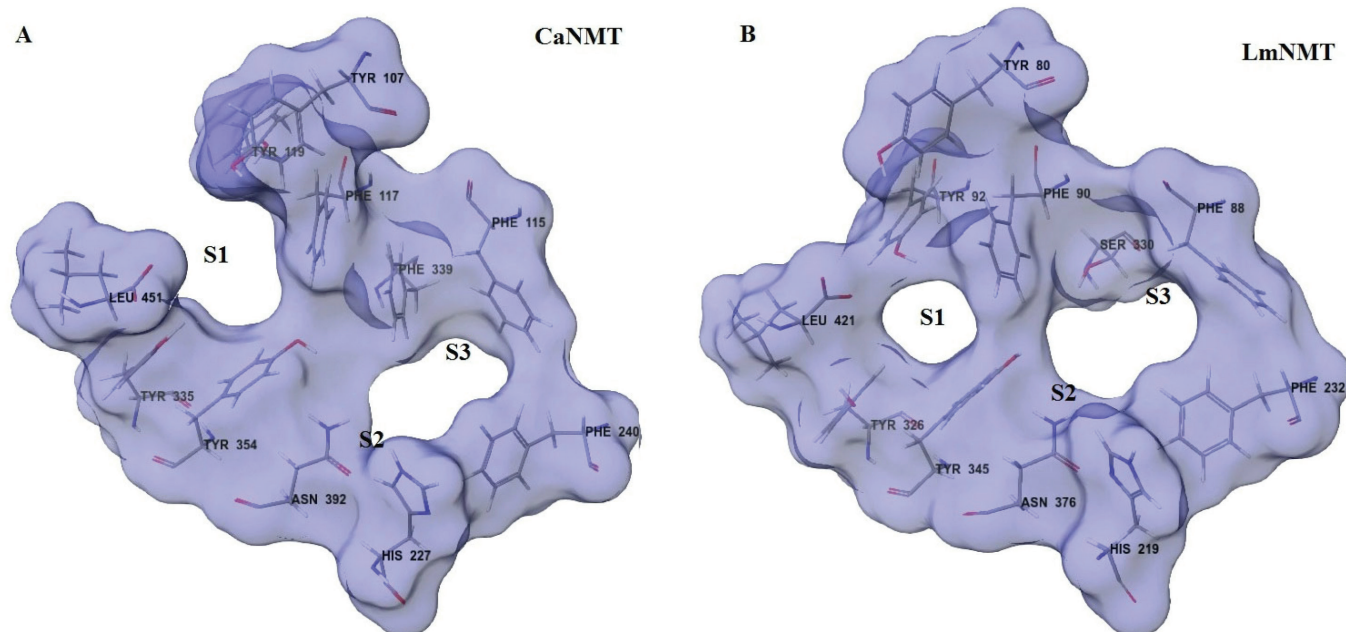
This comparative analysis of the crystallized active conformation of both enzymes was a useful guide to comprehend the differences between the two test compounds in the docking results.

Docking results of BF at CaNMT binding site reproduced the findings of Masubuchi *et al.* in 81% of the obtained docking poses. Scores of 87.830 (Goldscore) and 43.796 (Chemscore) were obtained to the best pose. This result validates our theoretical docking protocol, since BF exhibited excellent *in vitro* activity against CaNMT (Kawasaki *et al.*, 2003). Hydrogen bonds (H-bonds) can be observed between the benzofuran oxygen and the His227 as well as between the nitrogen from 2-pyridinyl ring at C<sub>4</sub> side chain and the Tyr119. Some poses have shown H-bonds occurring between the His227 and the oxygen from the C<sub>2</sub> chain side instead of the benzofuran oxygen, but this possibility was also observed by Masubuchi *et al.* (2003) (Figure 3A).

Pi-stacking interactions were established at both extremities of the molecule. The 2-pyridinyl ring interacts with Phe117 as well as the trifluorobenzene, at C<sub>2</sub> side chain, interacts both with Phe115 and Phe240. These results reinforce the importance of hydrophobic interactions at S3 pocket to the recognition by the CaNMT.

The BTZ docking at CaNMT, however, did not show the same profile. Only 55% of the 150 solutions met the established criteria of analysis and resembled the reference pose described by Masubuchi *et al.* Score values of 77.259 (Goldscore) and 34.412 (Chemscore) were obtained. When compared to BF, these results could indicate that BTZ might not be such a good ligand of CaNMT as BF.

The best pose, however, exhibited the same previously described crucial interaction points to good ligands (Figure 3B). H-bonds were observed between the



**FIGURE 2** - Comparison between the binding sites of CaNMT (A), the reference enzyme, and LmNMT (B).

nitrogen from C<sub>4</sub> side chain and the Leu421 as well as between the benzothiazole nitrogen and His227. Possible H-bonds with the oxygen from the C<sub>2</sub> side chain and this histidine were also observed to the BTZ. Similarly, pi-stacking interactions with the phenylalanine residues from S3 pockets were visualized, but no such interaction was observed at C<sub>4</sub> side chain.

Although Yamazaki et al. had synthesized benzothiazole analogues with excellent CaNMT inhibitory activity, none of them resembles the structure of BTZ herein studied. In fact, their best compounds present large groups at C<sub>6</sub> side chain of the benzothiazole system, which could possibly result from a better adjustment of them at S1 site of *C. albicans* enzyme. Our comparative analysis has revealed this site larger than the one from LmNMT and, consequently, their biological findings corroborate ours (Yamazaki *et al.*, 2005).

Regarding the LmNMT, molecular docking studies pointed out BTZ as a better ligand of this enzyme than its benzofuran analogue BF.

The reference pose was observed for the BF in only 30% of the solutions (Figure 4A) with score values of 61.794 (Goldscore) and 27.438 (Chemscore). The best pose presented, however, H-bond interactions in three distinct points: between the nitrogen from the C<sub>6</sub> side chain of BF and both Leu421 carboxylic oxygens as well as with the Tyr92 aromatic hydroxy group. Nitrogen from the 2-pyridinyl group has also established H-bond with the Tyr80. Pi-stacking interactions could be observed not only between the aromatic ring from C<sub>2</sub> side chain and Phe232

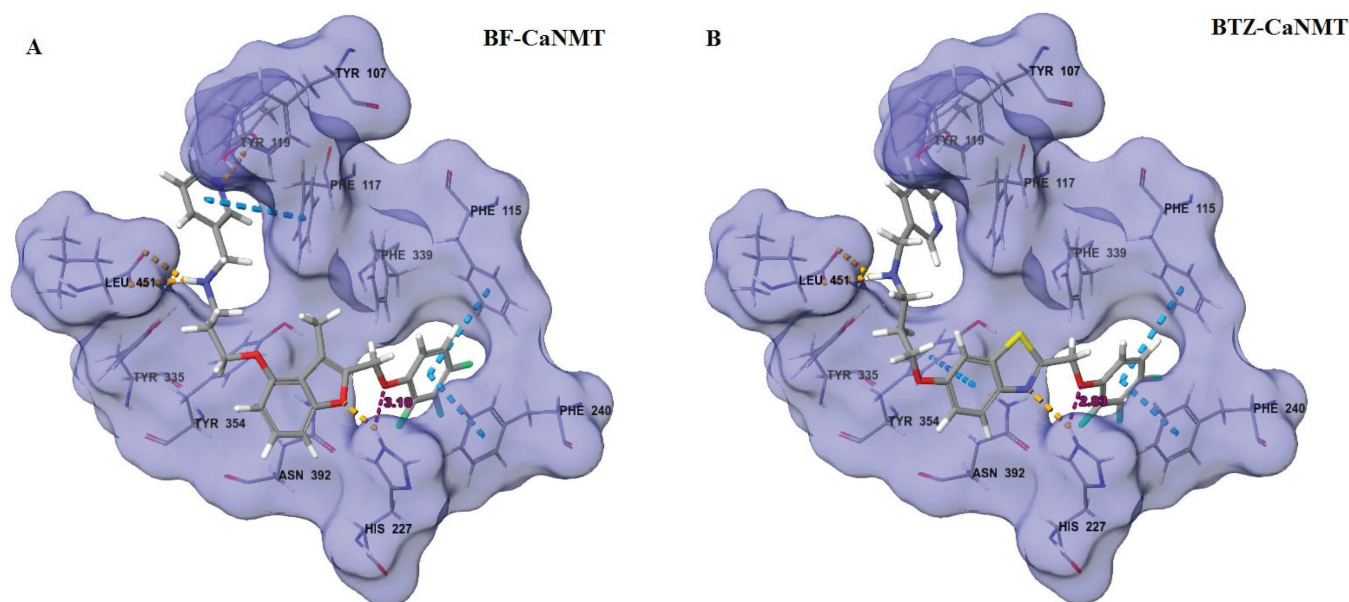
but also between the benzofuran ring and Phe90.

Neither the oxygen of the benzofuran ring nor the oxygen of C<sub>2</sub> side chain positioned itself close enough to established H-bonds with the His219. The closest distance achieved between these ligand groups and the enzyme is shown at Figure 4A and was of 3.15 Å.

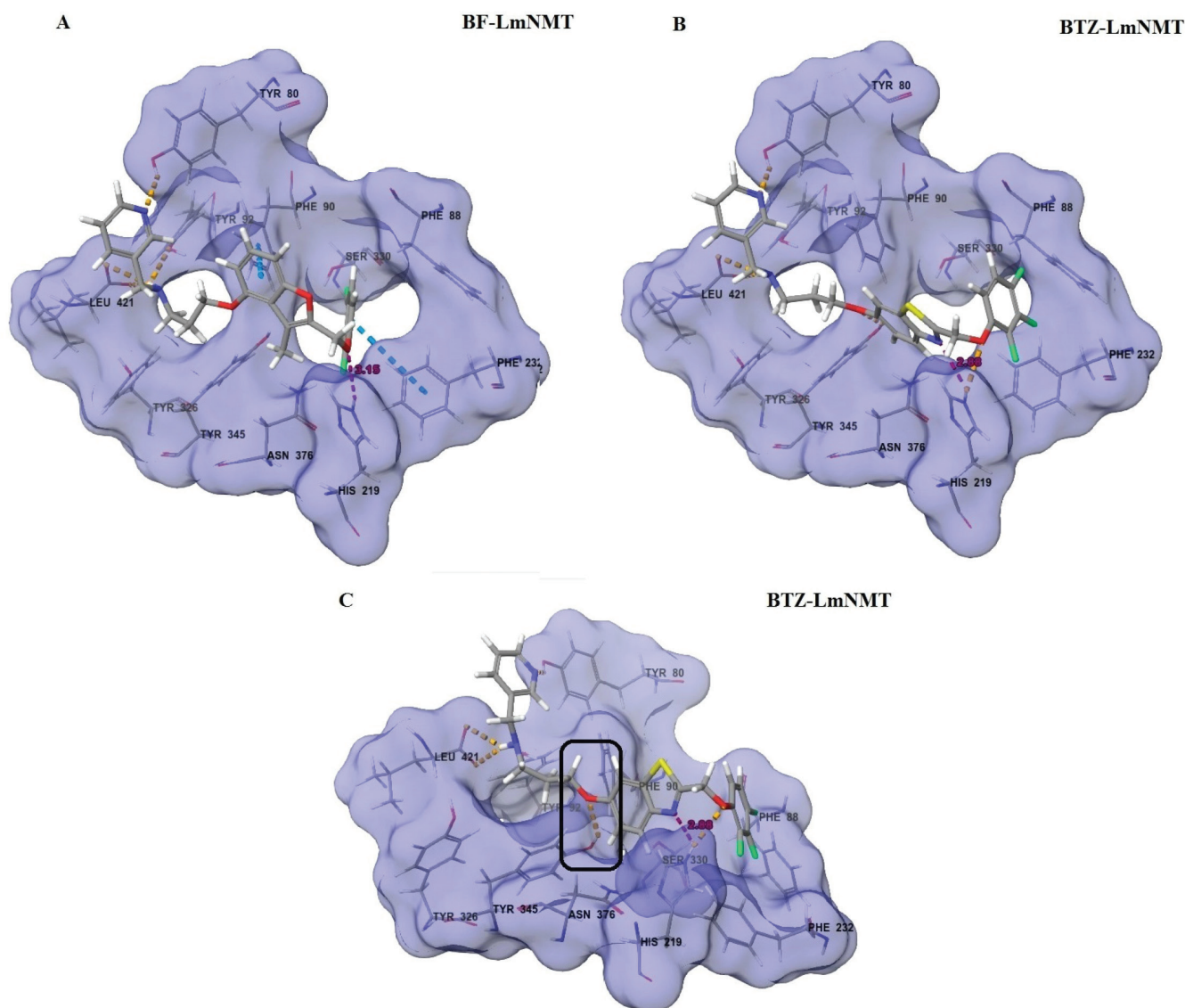
Despite this suitable pattern of interactions, bad steric contacts can be easily detected. The methyl group at C<sub>3</sub> position of benzofuran system, for instance, is found in an unsuitable proximity of Tyr345 and Asn376. A similar situation is observed with the positioning of the C<sub>4</sub> side chain carbons, which seem to assume a hard angle to fit into the S1 cavity. The steric salience formed by the residues Phe90 and Tyr345 at LmNMT could explain why BF assumed such a bad positioning, since this salience could be incompatible with BF's more rigid and hindered (by the methyl C<sub>3</sub> group) structure.

Our findings to BF are, thus, conflicting since the compound showed some poses with relevant score values associated to a suitable pattern of interactions. The low incidence of similar docked poses, however, could induce one to conclude that BF might not be a good ligand of LmNMT. The complex, however, could assume a more relaxed positioning when considering the dynamics of the system. For this reason, BF could not be neglected as a potential ligand of LmNMT.

BTZ, on the contrary, exhibited a high frequency of repeated poses, 80%, similar to the reference, with higher score function values, 74.453 (Goldscore) and 20.842 (Chemscore).



**FIGURE 3** - Docking studies with CaNMT. A. BF best docked pose; B. BTF best docked pose. Dotted lines represent the observed interactions. Yellow: H-bonds; Blue: Pi-stacking interactions; Magenta: potential H-bonds.



**FIGURE 4** - Docking studies with LmNMT. A. BF best docked pose; B. BTF best docked pose, view 1; C. BTF best docked pose, view 2. Dotted lines represent the observed interactions. Yellow: H-bonds; Blue: Pi-stacking interactions; Magenta: potential H-bonds.

The nitrogen from the C<sub>6</sub> side chain positioned itself close enough from the Leu421 to establish H-bonds with both carboxylate oxygens. His219 also interacts through H-bonds with the oxygen from the C<sub>2</sub> side chain. The nitrogen from benzothiazole ring is also close enough from the His219 to establish such interaction and, half of the best docked poses showed this interaction, instead of with the oxygen from C<sub>2</sub> side chain, as the most probable one (Figure 4B).

Finally, a fourth possible H-bond interaction could be observed, occurring between the Tyr345 and the oxygen from the C<sub>6</sub> side chain (Figure 4C).

These findings agree with what other authors observed.

Yu *et al.* (2012) also employing the Piggyback approach, identified a benzofuran analogue which could be recognized by the *Plasmodium falciparum* NMT (PfNMT). They synthesized a benzofuran structure very similar to that studied by Masubushi *et al.* and submitted it to crystallography analysis. Their results showed a ligand positioning very similar to the one observed in this study, with the oxygen of the benzofuran ring establishing H-bond interactions with the His213, the equivalent residue of the His219 from LmNMT. They also observed H-bonds between the His213 and the oxygen atom from the ester carbonyl group at C<sub>2</sub> of their benzofuran core. This oxygen occupies a similar positioning in this ligand to the one at C<sub>2</sub> from the BTZ herein studied (Yu *et al.*, 2012).

H-bond interactions with the terminal leucine residues and the neighbor tyrosine, in PfNMT Leu410 and Tyr107, were also observed both with the nitrogen from the 2-pyridinyl ring from C4 side chain. In the case of BTZ-LmNMT complex, however, these interactions were established by different atoms of the ligand, as already described.

In fact, several studies with NMT both from *P. falciparum* and *P. vivax* show these two residues as important in the recognition of ligands by the active site of these enzymes (Goncalves *et al.*, 2012; Yu *et al.* 2012; Rackham *et al.*, 2013; Rackham *et al.*, 2014).

BTZ presented good steric complementarity to LmNMT, with the trifluorobenzene ring at C<sub>2</sub> side chain pointed toward the most hydrophobic region of S3 cavity; however no Pi-stacking interaction was observed between this ring and the phenylalanine residues. Pi-stacking interactions, however, depend on the positioning of planar rings which, in turn, rotate freely. This means that, when considering the dynamics of the system, the trifluorobenzene ring can assume the right positioning to establish pi-stacking with the Phe88 and Phe232, as observed in the case of BF.

Previous homology and docking studies followed by molecular dynamics simulations revealed the topology of the binding sites from different parasitic NMTs, including the LmNMT. The authors described five important regions: two hydrophobic pockets, a hydrogen bonding pocket and two other pockets which also establish hydrogen bonding interactions, but one is negatively-charged while the other is positively-charged. Among the residues constituting one of the hydrophobic pockets in LmNMT model, the Phe88, Phe90, Phe232 were mentioned (Sheng *et al.*, 2009).

The positioning of BTZ trifluorobenzene ring towards these residues could be explained by the tendency of this moiety in interacting through hydrophobic forces in this region.

Moreover, the higher number of H-bonds established within BTZ-LmNMT, allied to the higher incidence of similar poses, configures BTZ as a potentially better ligand of this enzyme than BF and, consequently, a good prototype to optimize in searching for new antileishmanial agents.

## CONCLUSION

In conclusion, our results indicate that CaNMT and LmNMT are very similar in their residues arrangement of S1, S2 and S3 regions from the binding site, allowing to extrapolate the knowledge of CaNMT inhibitors to the development of new anti-LmNMT compounds.

BTZ was indicated as a good prototype to be optimized in the search for new inhibitors, while BF presented inconclusive results.

Due to the protrusion observed in the structure of LmNMT, less hindered and/or flexible ligands could present better docking behavior when regarding this enzyme, but studies of molecular dynamics could shed some light on this aspect.

Dynamics simulations might also improve the comprehension of aspects not observable through molecular docking studies, such as factors related to the essential induced fit phenomenon. For these reasons, such studies have already been initiated to the complexes herein discussed, particularly to BTZ-LmNMT.

BTZ presented good score functions values associated with interaction with residues previously reported as important to inhibitors recognition, such as with the His219, Leu421 and a predisposition to direct its most hydrophobic ring, the trifluorobenzene ring at C<sub>2</sub> side chain, toward the hydrophobic S3 region.

Finally, BF exhibited a suitable profile of molecular interactions with LmNMT, but only at 30% of the docking results. The compound, however, should not be discarded as a potential ligand of this enzyme since the same induced fit phenomena could help in ligand adjustment to the enzyme recognition site. Further studies should be performed to better understand the BF potential as a LmNMT inhibitor.

In general, the results herein discussed are in agreement with previously reported findings described to experimentally proved NMT inhibitors. This supports the potential of this benzoheterocyclic analogues as potential NMT inhibitors which might be explored in searching for new antileishmanial compounds.

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