N-Myristoyltransferase: A Novel Target

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Abstract: Myristoyl-CoA:Protein N-myristoyltranferase (NMT) is a cytosolic monomeric enzyme which catalyses the transfer of a rare fatty acid , myristate from myristoyl-CoA to the N-terminal glycine residue of a variety of eukaryotic and viral proteins. N-myristoyltransferase is a novel target for Anticancer, Antiviral and antifungal agents. Recent N-myristoyltransferase inhibitors like benzofurans and benzothiazole derivatives show *in vivo* antifungal activity and are promising selective fungal N-myristoyltransferase inhibitors.

Key Words: N-myristoyltransferase, myristoylation, benzofurans, benzothiazoles, antifungal, *Candida albicans,* anticancer, antiviral.

1. INTRODUCTION

In last two decades the incidence of cancer and viral infections has increased. Also the frequency of systemic fungal infection which are often life threatening has increased dramatically due to the increase in the number of patients who are immunocompromised by acquired immunodeficiency syndrome (AIDS), cancer chemotherapy or organ transplantation. Fungal infection range in severity from common superficial problems affecting the skin, nails, to deeply invasive and disseminated infection such as systemic candidasis and pulmonary aspergillosis. Currently there are four classes of antifungal drugs, Polyenes, Azoles, Flucytosine and Candins. However there use is restricted by their limited activity spectrum, toxicity, hazardous interactions and non-optimal pharmacokinetics [1]. Furthermore, important fungal pathogens are becoming resistant to these drugs.

To overcome the drawbacks of current drugs and to obtain more effective drugs, development of chemotherapeutic agents with a novel mechanism of action is essential. One such target that has been identified recently for the development of antifungal agents is Myristoyl-CoA:Protein N-myristoyltransferase (NMT).

2. MYRISTOYL-COA: PROTEIN N-MYRISTOYLTRANSFERASE -A NOVEL TARGET FOR CHEMOTHERAPEUTIC AND ANTIFUNGAL AGENTS

N-myristoyltranferase is a cytosolic monomeric enzyme [2,28] and are present in eukaryotes like protozoa, fungi and animals but are absent in bacteria. N-myristoyltranferase catalyzes a reaction called myristoylation which involve the transfer of myristate from myristoyl-CoA to the substrate protein. This is very essential for full expression of biological function of the cellular and viral proteins [3,34] and in malignant transformation of cancerous cells. Since myristoylation is important for full expression of certain oncogenes (eg. $pp60^{\delta TC}$) and proteins necessary for virus particle as sembly (eg. gag polyprotein precursor of HIV-I), N-myristoyltranferase can be selected as a novel target for the design of potential antineoplastic [5,33] and antiviral agents [4,6].

In fungi, the myristoyl group is considered to be involved in the regulation of interaction between a myristoyl-protein and cellular membrane. These proteins are involved in signal transduction cascade and vesicular and protein trafficking. For example, in *S. cerevisiae* NMT catalyzes productions of twelve N-myristoyl proteins [7], four of these are very essential for full expression of their biological activity. 1) Vps15p a serine/threonine kinase required for vacuolar protein sorting. 2) Gpa1p required for it to act as a negative regulator of the mating pathway. 3) Arf_1 and Arf_2 proteins (ADP ribosylation factor). 4) 21-KDa GTP binding proteins involved in vesicular and proteins trafficking.

C. albicans with defective NMT looses the ability to infect mice [8]. Genetic studies have also established that the enzyme is essential for survival of *C. albicans* and *C. neoformans* which are important pathogenic fungi [9,10]. Also, it is proven that NMT is essential for viability of yeast [11]. Furthermore, fungal and mammalian NMT differs in the peptide substrate specificity; hence inhibitors are relatively specific for fungal enzyme [12]. The necessity of N-myristoyl protein, the production of which is catalyzed by the enzyme NMT, in various fungi, eg *Candida albicans* and *Cryptococcus neoformans* which causes systemic fungal infection makes NMT a suitable target for antifungal agent.

3. MYRISTOYLATION

Myristoylation refers to cotranslational attachment [13] of myristic acid, a 14-C saturated fatty acid, to the N- terminal Glycine residue of proteins *via* an amide bond Fig. (**1**). Myristoylation is generally irreversible and is a very important lipid modification at the N-terminus of eukaryotic & viral proteins. It is involved in directing and anchoring proteins to membranes and as consequence, cellular regulation, signal transduction, translocation, several viral induced pathological processes and apoptosis [14,15]. Attachment of myristoyl residue provides hydrophobicity to influence the

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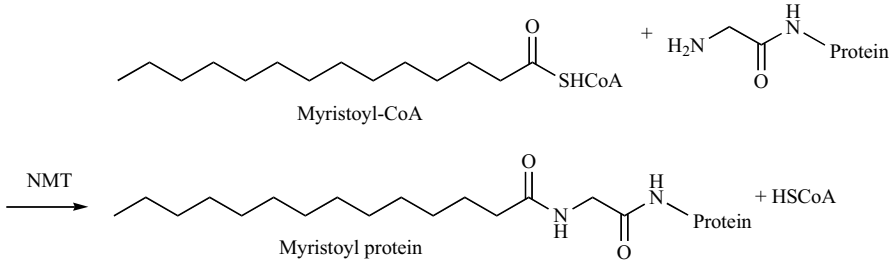


Fig. (1). The process of myristoylation.

partitioning of proteins to cellular membranes and serve to promote protein-protein interaction [16].

4. THE CATALYTIC MECHANISM OF NMT: AN ORDERED BI BI REACTION MECHANISM

Catalysis by NMT occurs *via* a sequential ordered Bi Bi mechanism [17] Fig. (**2**). The enzyme first forms a Myristol-CoA – NMT binary complex with high selectivity for myristoyl-CoA. This complex influences interaction of NMT with peptide. A peptide substrate then binds to generate a Myristoyl-CoA-NMT-peptide ternary complex. Following the catalytic transfer of myristate from CoA to peptide substrate, free CoA is released first and the N-myristoylated protein second.

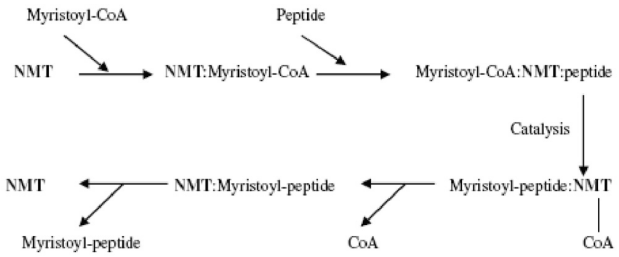


Fig. (2). The catalytic mechanism of NMT.

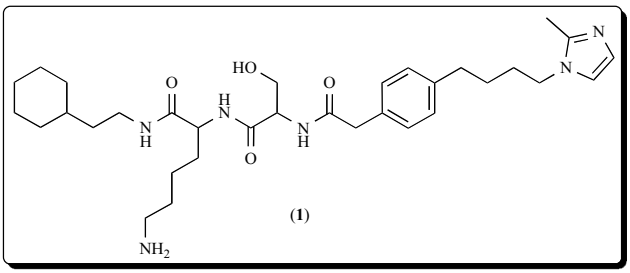
5. *C. ALBICANS* N-MYRISTOYLTRASFERASE (CANMT) AND ITS INHIBITOR BINDING SITES

Protein N-myristoyltranferase is a member of the GCN[5] acetyl transferase (GNAT) super family [18]. Structure of enzymes belonging to this family consist of a N-terminal strand (β_1) followed by two helices (α_1 & α_2), three anti parallel β strand (β_1, β_2, β_3), followed by a 'signature' central helix (α_3), a fifth (β_5) strand, a fourth α helix and a final β strand (β_6). *Candida albicans* N-myristoyltranferase (CaNmt) consist of 451 amino acid residue [12] and NMT1 of *S. cerevisiae* has 455 residues [19,20], where as human N-myristoyltranferase (hNmt) is a 416 residue proteins encoded by a single copy gene [21].

The N-terminal half forms most of the Myristoyl-CoA binding site, while the C-terminal half contributes largely to the peptide binding site. Below is explained how a peptide and a non peptide NMT inhibitor binds to the enzyme [18].

5.1. Binding Mode of a Peptide Inhibitor (1)

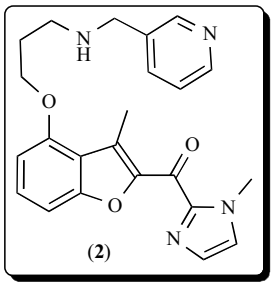
The inhibitor lies in an extended conformation in a long groove formed by β strands and loops of residues 238 to 249 of the C-terminal half and residues 106 to 115 of N-terminal half. Moreover, the bound myristoyl-CoA itself forms a part of binding site for the inhibitor. The 2-methylimidazole group mimics the N-terminus of peptide substrate and interacts with carboxyl group of the C-terminus residue (Leu451). The aliphatic chain between imidazole ring and the benzene



ring is bent and is in contact with myristoyl-CoA, thus guiding the imidazole ring to the C-terminus carboxylate. Several hydrophobic residues, Val108, Phe117, Tyr225, and Phe339, make van der Waals contacts with the benzene ring. The hydroxyl group of serine is hydrogen bounded to the side chain imidazole group of His227 and to the main-chain amide group of Gly413. The amino group of lysine is surrounded by the side chain of negatively charged residues Asp110, Asp112, and Asp412. The aliphatic moiety of lysine is covered by the aromatic side chains of Phe115 and Phe240. The peptide moiety is also bound to the main chain amide nitrogen and the side chain carboxyl group of Asp412. The 2-cyclohexylethyl amide interacts with Pro229 and Tyr256.

5.2. Binding Mode of Nonpeptide Inhibitors (2), A Benzofuran

The non peptide inhibitors are situated in the region of the substrate binding site, but with different interactions from those of a peptide inhibitor. The benzofuran moiety is located at the center of a deep pocket, surrounded by hydrophobic residues. The pocket of the inhibitor binding site is composed of aromatic residues Tyr107, Phe115, Phe117, Tyr119, Phe176, Tyr225, Phe240, Tyr256, Tyr335, Phe339, and Tyr354. The benzofuran is stacked parallel to Tyr225 and perpendicular to Tyr354 in the proximity of Phe117 and Phe339. These residues are important not only for the architecture of binding site but also for inhibitor binding. His227 is located in proximity of the oxygen atom of benzofuran ring which interacts through hydrogen bond and is essential for geometry. The secondary amine group of the substituent



(**2**)

at position 4 of the benzofuran ring makes a hydrogen bond with C-terminus caboxylate. The secondary amine is essential for inhibitory activity. In the complex with compound, the methyl imidazole ring makes a hydrogen-bond interaction with the side chain of Asn392 and is positioned in close proximity to Phe240. A bulky substituent at position 2 is essential for enzyme inhibition.

Due to the binding of inhibitor conformational changes takes places in the enzyme structure and results in enlargement of the binding site and interaction with the secondary amine of the nonpeptide inhibitor. A rearrangement of the side chain of Tyr225, accompanies nonpeptide inhibitor binding. Structural adjustments of aromatic residues Phe117, Tyr119, and Tyr354 are observed. These conformational changes indicate that the peptide substrate is trapped by the closure of the enzyme in the catalytic reaction and that the non peptide inhibitor binds deep in the binding site and thus prevent peptide substrate binding.

5.3. Selectivity for Inhibitors

A number of inhibitors exhibit high inhibitory activity against fungal Nmt with low inhibitory activity against hNMT [22-25]. Such high selectivity is good for an antifungal agent. The myristoyl-CoA binding site of purified Human NMT and fungal NMT are highly conserved [10,26], but their peptide-binding sites are divergent [10,21,26-28], a fungal inhibitor should therefore target the enzyme at the peptide binding site to avoid toxicity. All Nmt have similar polypeptide folding. There are, however, a few specific amino acid differences in the inhibitor binding site between CaNmt and hNMT. For example, Phe339 and Lle352 residues of CaNmt are replaced by serine and alanine, respectively, in hNMT. Leu451 is replaced by glutamine in hNMT [18]. High selectivity of the inhibitor might be caused by these differences of surrounding residues.

6. NMT AS TARGET FOR ANTIFUNGAL AGENTS

As mentioned previously, N-Myristoyltransferase performs an important role in the myristoylation of vital proteins and genetic studies have established that NMT is essential for the growth and survival of various fungi. Therefore fungal NMT is a good target for the development of antifungal agents with a new mode of action. Five classes of NMT inhibitors are reported. Fig. (**3**) 1) Peptidomimetic inhibitors [2,27], 2) Myristic acid analogs [29,30], 3) p-toluene sulphonamide inhibitors [31], 4) Benzofurans inhibitors[22-24], 5) Benzothiazole inhibitors[25]. In this review article we have discussed about benzofurans and benzothiazoles derivatives in detail as these structures are promising in terms of activity and selectivity. Compound (**3**) is the first potent and selective fungal NMT inhibitor that was reported in 1995 by Devadas *et al.* [27]. This peptidomimetic inhibitor was designed based on the substrate peptide GLYASKLS-NH_2 that was derived from the N-terminal fragment of Arf2p and its analogous peptide inhibitor ALYASKLS-NH_2. However the peptide and peptidomimetic inhibitors of CaNmt, due to their peptidic nature are devoid of *in vitro* antifungal activity.

Since no peptidomimetic inhibitors showed strong antifungal activity, the starting octapeptide (ALYASKLS) was modified by to get a new class of nonpeptide compound [32]. In this, the 1st four residues ALYA is replaced by a *p*-[(2-methylimidazole-1-yl) butyl phenyl]-acetyl group in which the imidazole moiety represents the key N-terminal recogni-

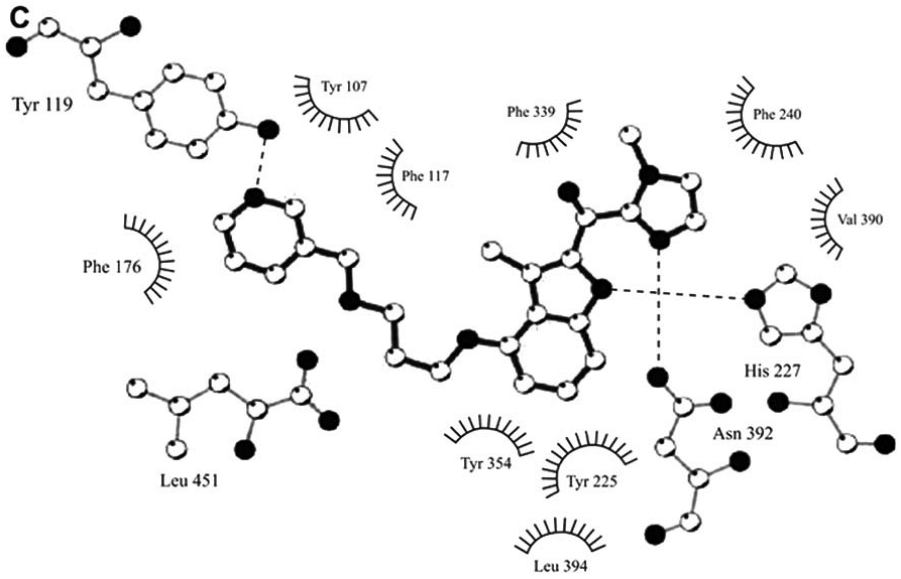


Fig. (3). A schematic drawing of interaction of a Benzofuran compound with the enzyme.

tion element alanine. The C-terminal tetra peptide SKLS is replaced by a chiral tyrosinol scaffold which retains the serine alcohol recognition element. The hydrophobic interaction fulfilled by leucine is represented by the cyclohexylethyl ether group. Compound (4a) is fungistatic against *C. albicans* whereas compound (4b) showed antifungal activity *in vitro* against *C. albicans* with an ED_{50} value 49μM. This compound was determined to fungicidal (MFC=150μM).

Antifungal activity of myristic acid analogs was tested by Parang *et al* [30]. In this (±)-2-Bromotetradecanoic acid (**5**) showed antifungal activity *in vitro* against *C. albicans, C. neoformans, S. cerevisiae and Aspergillus niger*. However no antifungal activities *in vivo* of these compounds are reported in the literature.

A series of p-toluene sulphonamides was synthesized by Karki *et al* [31] based on the molecular modeling studies. Some of them showed weak antifungal activity against *C. albicans* ATCC24433. However no NMT inhibition data of these compounds were reported.

6.1. Benzofurans As Fungal NMT Inhibitors

Although several peptidomimetic inhibitors of CaNmt were synthesized and tested, their antifungal activity was only marginal. A random screening of the Roche chemical libraries by researchers led to the discovery of lead compound (**7**). It competitively inhibited CaNmt (IC_{50} 0.98μM) with high selectivity over human NMT (IC_{50} 194μM).

The *in vitro* antifungal activity of (**7**) is marginal and it also shows β-adreno receptor blocking activity because of hydroxyl group on the side chain at 4th position. SAR led to the development of more potent structures with benzofuran nucleus Fig. (**4**).

1) The trimethylene group is the optimum side chain length at the 4th position.

2) A hydrophobic or aromatic alkyl group is necessary to interact with hydrophobic aromatic amino acid residues, Tyr-107, Tyr119, and Phe176. Compound (8) having a (pyridin-3-ylmethyl) amino in place of t-butyl group, is 10 times more potent than (7) in terms of enzyme inhibitory activity and with high selectivity (>5000 fold) over human NMT [22].

3) However (**8**) did not show *in vivo* efficacy in a murine systemic candidiasis model because the ester group of (**8**) is easily hydrolyzed by esterase in the mice to give an inactive carboxylic acid metabolite.

4) To overcome this problem the ethoxycarbonyl group at 2-position was modified. To strengthen the binding between the C-2 substituents and the phenylalanine residues by aromatic-aromatic interaction, a phenyl group was introduced to the 2-position *via* various linkers: -CONH, $-CH_2S$, $-CH_2CH_2$, $-CH_2O$. In which $–CH_2O-$ was identified to be the best in terms of stability, enzyme inhibitory activity and antifungal activity.

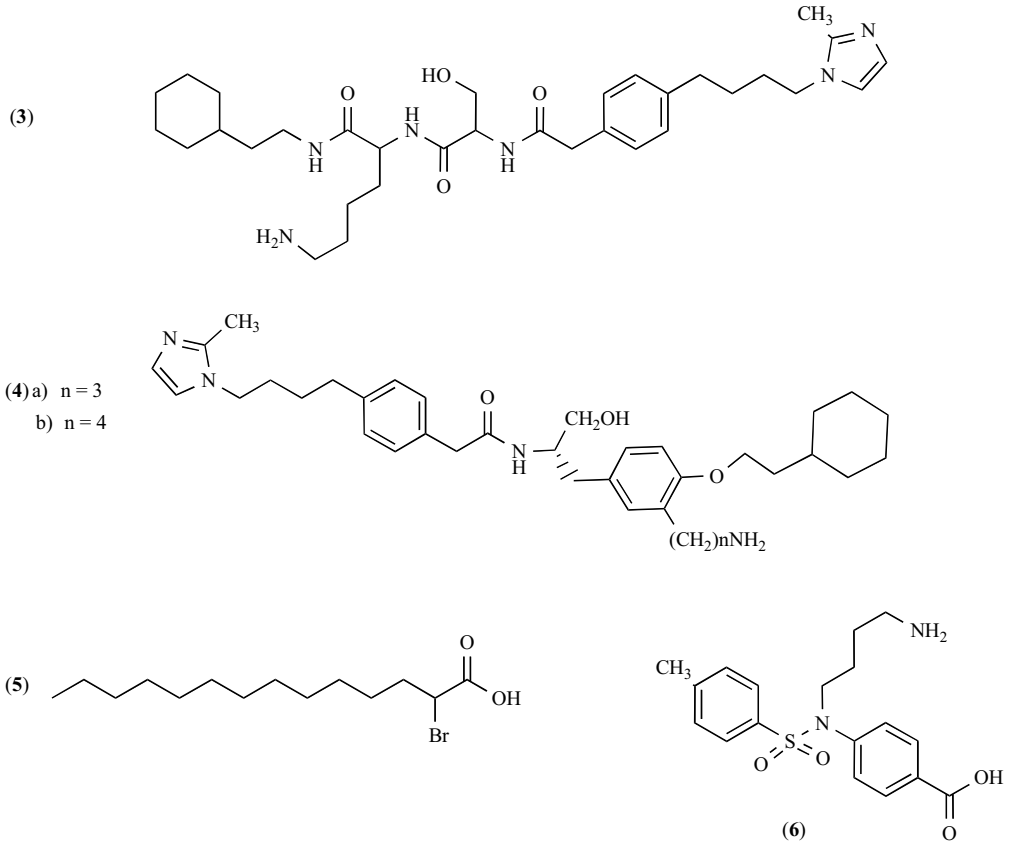


Fig. (4) Structure of some NMT inhibitors.

5) To further increase the binding affinity, electron withdrawing groups such as fluoro and cyno groups is introduced to the phenyl ring. This increased the activity significantly. The compounds show good pharmacokinetic profile compared to (**8**). In this series, compounds (**9**) and (**10**) exhibited *in vivo* antifungal activity and showed relatively long $t_{1/2}$ [13].

6) However the two compounds (**9**) and (**10**) were not stable in artificial gastric fluid (pH 1.2) and had less activity against *Aspergillus fumigatus*. Acid instability was due to arylmethyl ether moiety and hence it is replaced by an acid-stable keto group (**11**). The nitrogen atom of pyridine makes a hydrogen bond with Asn392 and pyridine ring fits into the hydrophobic pocket. Introduction of methyl group (**12**), an electron donating group, on pyridine ring increases the electron density on pyridine ring and hydrogen bond interaction with Asn392 and thus increases the potency. Compound (**12**) is most potent in this class of acid stable benzofuran Nmt inhibitor [24].

Thus benzofuran Nmt inhibitors are the required leads for the development of antifungal agents, which can eliminate life threatening infections such as systemic candidiasis.

6.2. Benzothiazole Derivatives As Antifungal NMT Inhibitors

Benzothiazoles have developed as most promising and selective fungal NMT inhibitors. (1R,3S)-N-{2-[(cyclopeantlycarbonyl)amino]-benzothiazole-6-yl}-3-[(2-naphthyl-methyl) amino]cyclohexanecarboxamide (FTR1335) is an example of a benzothiazole derivative, the chemical structure of which is given below Fig. (**5**).

FTR1335 strongly inhibits CaNmt in a dose dependent manner with an IC_{50} of 0.49±0.04nM. Conversely, FTR1335 showed a little dose dependent inhibition of HsNmt1 with an IC_{50} of 5400±260nM, indicating an excellent selectivity over 10000X between 0.49 and 5400.

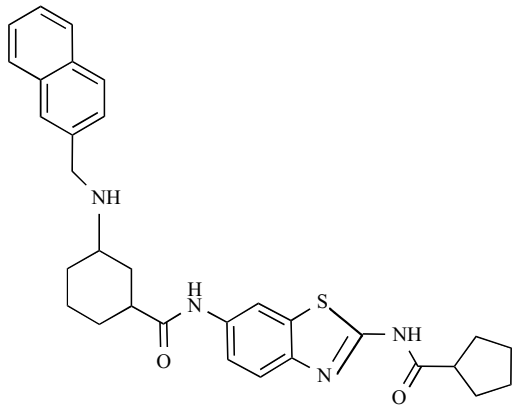


Fig. (6). Structure of FTR1335.

As mentioned previously the enzyme has an ordered Bi Bi reaction mechanism. FTR1335 is competitive with the

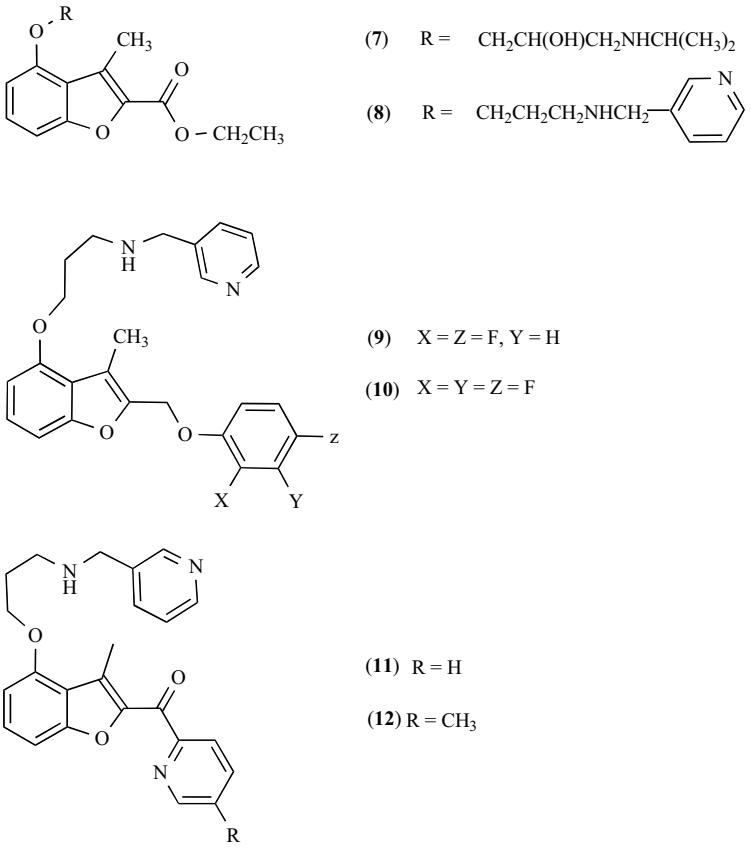


Fig. (5). Some benzofuran derivatives as fungal NMT inhibitors.

peptide binding site, but non-competitive with the myristoyl-CoA binding site [25]. This indicates that FTR1335 acts at only the peptide binding site of CaNmt, suggesting that it is an appropriate lead compound for further development. FTR1335 also has good antifungal properties *in vitro*.

Thus FTR1335 is a suitable platform for developing novel antifungal agents, as the benzothiazole structure offers the most promise in terms of selectivity and fungicidal activity.

7. NMT AS TARGET FOR ANTINEOPLASTIC AGENTS

Colorectal cancer is a major cause of death, particularly in the western world, leading to 400,000 deaths each year [5]. NMT as a target for cancer therapy is a new approach and lots of research is expected on this project. Myristoylation is one of the important mechanisms by which a protein associates with membrane and these cellular myristoylated proteins have diverse biological functions in signal transduction and oncogenesis. Examples include the catalytic subunit of cAMP[4]- dependent protein kinase, various tyrosine kinases ($pp60^{src}$, $pp60^{yes}$, $pp56^{lck}$, $pp59^{fyn/syn}$ and c-Ab1), the β-subunit of calcineurin, the α-subunit of several guanine nucleotide binding proteins and ADP ribosylation factors [33-35].

NMT is more active in colonic epithelial neoplasm than in the corresponding normal colonic tissue and this increase in the NMT activity occurs in early stages of colonic carcinogenesis. Hence NMT can be used as a potential marker in early detection of cancer. An increased NMT activity was observed in human colonic tumors [33,36]. Also, increased expression of NMT was observed in gall bladder carcinoma [37]. These finding are significant with respect to design of chemotherapeutic drugs. Cell transformed by oncogenic protein tyrosine kinase, such as $pp60^{src}$ show change in cell growth and cell morphology. Normal intestinal epithelial cell express high levels of myristoylated $pp60^{src}$ and $pp60^{c\text{-}yes}$ [38]. Blockage of myristoylation of pp60src in colonic cell lines depressed colony formation, cell proliferation and localization of $pp60^{src}$ to the plasma membrane [5]. This provides evidence that myristoylated protein are involved in the pathogenesis of cancer and candidates which inhibit NMT may be therapeutically useful.

Recently Shrivastav *et al.* [33] discovered enolase, a glycolytic enzyme, as a potent inhibitor of the myristoylation reaction *in vitro*.

Examples of compounds used to inhibit NMT activity (Fig. **7**).

1) Mannich bases of α, β-unsaturated ketone have some cytotoxic activity [39].

2) The cysteine 169 thiol group of NMT is involved in forming covalent bonds with the substrate [40]. Hence thiol alkylators are under research as NMT inhibitors [5].

8. NMT AS TARGET OF ANTIVIRAL AGENTS (ANTI-HIV-1)

Human immunodeficiency virus (HIV) is the causative agent of AIDS in humans. The main structure components of

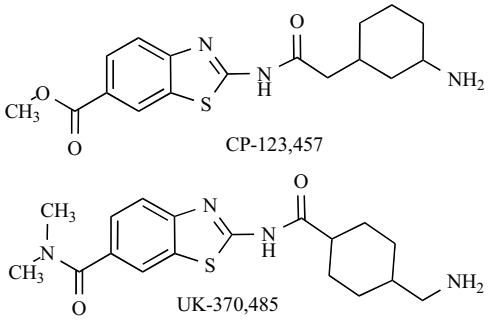


Fig. (7). Examples of benzothiazole derivatives.

HIV particles are encoded by the gag gene, and expression of Gag protein is essential for production of viral particles.

NMT plays a very important role in HIV-I, because the N-termini of viral proteins, $p17^{gag}$ and Nef, are N-myristoylated which is necessary for conferring infectivity [41-43] and the effective replication of HIV-I. Therefore, human NMT (hNMT) is a potential target of anti-HIV-I agents. But at the same time, inhibition of hNMT activity may damage the host cell. Also, myristoylation of HIV-1 gag protein is essential for virus particle budding [44].Compounds which have been reported to inhibit HIV replication and are expected to inhibit NMT activity are hetroatom-substituted analogs of myristic acid [45,46], phosphorous containing such a myristic acid analogue [47], and analogues of N-myristoyl glycine [48,49].

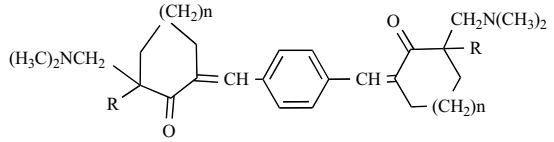
Examples of serinal derivatives [50] which are under research as NMT inhibitors are-

1) O-myristoyl serinal bisulfite.

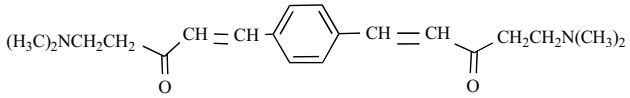
2) N-acetyl-O-myristoyl serinal diethyl acetate.

3) N-myristoyl serinal bisulfite.

The above observation suggest that myristoylation of retrovirus gag protein is a potential target for development of novel anti-viral agents.



(**10**)
a: R = H ; n = 0, b: R = CH3 ; n= 1
c: R = H ; n = 2



(**11**)

Fig. (8). Structure of some NMT inhibitors.

9. NMT AS TARGET FOR ANTI-PARASITIC AGENTS

N-Myristoyltransferase is a essential enzyme in kinetoplastod protozoan parasites, *Leishmania major* and *Trypano-*

soma brucei . *L. major* NMT myristoylates proteins such as ARF (ADP ribosylation factor)-like proteins 1 and the infective stage specific, hydrophilic acylated surface proteins (HASPs). It is reported that NMT activity is essential for viability of in *Leishmania.* Genetic studies also show that expression of the *T. brucei* NMT gene is essential for viability in both vector (procyclic) and mammalian (bloodstream) parasitic stages [51]. Myristate analoges, non specific inhbitors of NMT, have been tested as anti-parasitic. 2-Hydroxymyristate and 4-Oxotetradecanoate inhibits the growth of *L. major*. The above observations clearly suggest that NMT may be appropriate targets for the development of antiparasitic agents.

CONCLUSION

N-Myristoyltransferase (NMT) inhibitors are new class of drugs. Clinically they are useful against Azole resistant *C. albican* diseases. In addition, since its mechanism is different from that of Azoles, it is also possible to overcome the problem of drug-drug interaction. Current antifungal agents are not ideal in terms of efficacy, antifungal spectrum and safety. NMT inhibitors are also selective in action therefore there is very low incidence of side effects. Thus, NMT is a novel target for the next generation of not only antifungal agents but also for anticancer, antiparasitic and antiviral agents. In future, lots of drugs targeting the enzyme N-Myristoyltransferase are expected.

ABBREVIATIONS

NMT = Myristoyl CoA-Protein N- myristoyltransferase.

CaNmt = *Candida albicans* N-myristoyltranferase.

hNmt = Human N-myristoyltranferase.

HIV = Human immunodeficiency virus.

AIDS = Acquired immunodeficiency syndrome.

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