African Journal of Biotechnology Vol. 8 (13), pp. 3072-3078, 6 July, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Virtual high screening throughput and design of 14α-lanosterol demethylase inhibitors against *Mycobacterium tuberculosis*

Hildebert B. Maurice[1]*, Esther Tuarira[1] and Kennedy Mwambete[2]

[1]School of Pharmaceutical Sciences, Institute of Allied Health Sciences, Muhimbili University of Health and Allied Sciences, Tanzania.
[2]Department of Pharmaceutical Microbiology, School of Pharmacy, Muhimbili University of Health and Allied Sciences, Tanzania.

Accepted 26 February, 2009

The current treatment against *Mycobacterium tuberculosis*, the causative agent of tuberculosis in humans, requires a drug combination and the last two decades have passed without significant development of novel chemicals for the treatment of tuberculosis. The elucidation of the sequence of genomes for *M. tuberculosis* has identified a gene that encodes a protein with 34% amino acid sequence similarity to human CYP51 which is referred to as MT CYP51. Azole compounds which inhibit fungal CYP51, also inhibited the growth of *Mycobacterium bovis* and *Mycobacterium smegmatis*, at nanomolar concentration. In this study, over 10,000 ligands from the NCI database were virtually screened for their free binding energy against mycobacterial 14α-lanosterol demethylase by docking. Ten hits which bound the enzyme at lowest free energy ranging from -13 to -14.5 Kcal/mol where selected. Various fragments from selected ligands were incorporated together to generate new lead compounds that bind the enzyme at the energy three times lower than fluconazole. To prove the concept, literature search on the inhibitory concentration at 90% (IC_{90}) demonstrated that ligands which were selected had activity against the *M. tuberculosis* which correlated well with binding free energy with few exceptions.

Key words: Docking, high throughput screening, *Mycobacterium tuberculosis*, ligands, 14α-lanosterol demethylase and IC_{90}.

INTRODUCTION

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis* is estimated to infect about one-third of the world's population, still remains the world-wide main cause of death among the infectious disease and kills about three million people annually (Aziz et al., (2006). The emergence of multidrug resistant strains (MDR) of *M. tuberculosis* coupled HIV/AIDS infection (Madariaga et al., 2008) which exposes patients to other opportunistic infections caused by atypical mycobacteria resulting in the *Mycobacterium avium* complex (MAC) in immunocompromised individuals prompts the search for new anti-mycobacterial agents (Girón et al., 2008; Hosaka et al., 2006).

The current treatment against *M. tuberculosis*, the causative agent of tuberculosis in humans, requires a drug combination under the directly observed treatments (DOTS) (Mishra et al., 2006; Wandwalo et al., 2006) and the last two decades have passed without significant development of novel chemicals for the treatment of tuberculosis. The AIDS epidemic and the emergence of drug-resistant *M. tuberculosis* strains have resulted in resurgence of this deadly disease.

Collaborative efforts have resulted in the elucidation of the sequence of genomes for *M. tuberculosis* and this has identified a gene that encodes a protein with 34% amino acid sequence similarity to

*Corresponding author. E-mail: hildebert1@yahoo.com. Tel: +255 717 399 304.

human CYP51 which is referred to as MT CYP51 (Jackson et al., 2003).

Azole compounds which are potent anti-mycotic agents, have been firmly established that their anti-mycotic properties results from their ability to inhibit CYP51 which reduces sterol levels, thereby weakening membrane structures and preventing cell growth (Guardiola-Diaz et al., 2001).

Using radio-labelled incorporation from mevalonate it has been shown that sterol biosynthesis occurred during growth of the organism even though at lower levels than in fungi and their importance is not resolved since hopanoids are the ones present in prokaryotic cells than sterols (Podust et al., 2001).

14α-Lanosterol demethylase (MT CYP51) catalyzes an essential early step in sterol metabolism whereby it removes a methyl group, from lanosterol. This leads to an accumulation of methylated sterol precursors. Inhibition of this enzyme leads to the disruption of cell membrane which results in inhibition of growth and/or cellular death (Micheal, 2006).

Recent studies have confirmed that azole derivatives which inhibit fungal CYP51, also inhibits the growth of *Mycobacterium bovis* and *Mycobacterium smegmatis*, accepted models for the study of *M. tuberculosis* at nanomolar concentration and were more active than the most used drug isoniazid and other inhibitors were identified experimentally through high throughput screening (Guardiola-Diaz et al., 2001).

It is therefore logical to think that developing inhibitors against the mycobacterial demethylase enzyme has the potential to kill *M. tuberculosis* and therefore a potential target for the discovery of new drugs to treat tuberculosis. More important, it has been proven that drug resistance existing in *Candida albicans* cannot cross to the mycobacterium due to the fact that in residues lining cavities the CYP51 enzyme of these two microorganisms are different and are coded by two different genes (Podust, et al., 2001).

METHODOLOGY

The design of enzyme binders was carried out first by docking ligands from the library and then by adding moieties on some of the identified hits using the docking programme, ArgusLab (Planaria Software LLC, Seattle, WA, USA). Compounds were accessed from two NCI database one containing 2,341 and the other Kegg containing 10,005 compounds and the mycobacterial 14α-lanosterol demethylase (Code 1H5Z) was obtained from RCSB Protein Data Bank (http://www.rcsb.org/pdb/2006). The whole enzyme was assumed to be the binding pocket to enable possibilities of some ligands to bind to other non-substrate binding pockets that exist which may identify important bindings and all bound waters and ligands were removed from the protein. So, the binding pocket size was calculated to be 60 x 55.515 x 60. All ligands were visually inspected and over 500 were selected, drawn, geometrically optimized, energy minimized and docked manually into the enzyme. ArgusDock was the docking engine and the ligand was set to be flexible with the Ascore as the scoring function at regular precision. Grids were precalculated automatically by the programme at the resolution of 0.400. Before docking, the numbers of ligand torsions are calculated and maximum number of poses was set to be 150. Root node radii for each ligand were calculated, poses were searched and energy for each was calculated and the ligand pose with the lowest energy was considered to be the best. At the end of docking, the ligand residue was highlighted and neighbouring amino acid and/or heme residues within 3.5 angstroms were selected. The programme was commanded to hide the unselected residues so as to determine amino acids lining the binding cavity. Also, hydrogen bonds and bumps were monitored. Importantly the distance between every ligand and heme was also determined.

The IC90 values on the whole organism were searched from the results that were published by Southern Research Institute through their website (www.taacf.org) in 2006.

RESULTS AND DISCUSSION

Ten hits with significantly lower binding free energy were identified with some that are already in clinical use to treat other diseases. Six of them bound the enzyme with free energy twice less than that of fluconazole. Also, leads generated had better binding features and even much lower binding free energy than azoles. The lower binding free energy is an indicator of the capacity of compound to inhibit the enzyme at lower concentrations (IC50). Some of the compounds that have the potential to inhibit the enzyme

From this study, it has been learnt that there are about fifteen residues essential for ligand binding namely 256 Ala, 101 and 259 His, 82 and 321 Ile, 79 Met, 433 Met, 78 Phe, 83 Phe, 255 Phe, 252 Ser, 176 Thr, 76 Tyr and 434 Val. These residues were considered to be neighbouring the ligand as long as it was in its 3.5Å proximity. At the base of the binding pocket, there is a heme porphyrin that accounts for some binding free energy of some ligands. With the exception of two ligands, ketoconazole and ligand 245, all ligands interacted with the active site. Ketoconazole and ligand 245, even though having an imidazole ring in common, interacted with the other site opposite of the validated one and this may suggest the possibility of generating non competitive inhibitors to natural ligands. The residues lining the ketoconazole pocket included 397 and 398 Ala, 389 and 393 Arg, 377 Asp, 308 Glu, 390 Gly, 401 Ile, 311 and 315 Leu, 312 Lys, 387 Phe, 386 Pro, 382 and 384 Trp (Figure 1).

Ligands that had the scoring function energy higher than-13 kcal/mol were not considered further. The other factor that was considered was the possibility of the docked ligand to interact with the heme. It was generalized that the ligand was in interaction with the heme if the later was found within 3.5Å distance. It was important because heme interaction is associa-

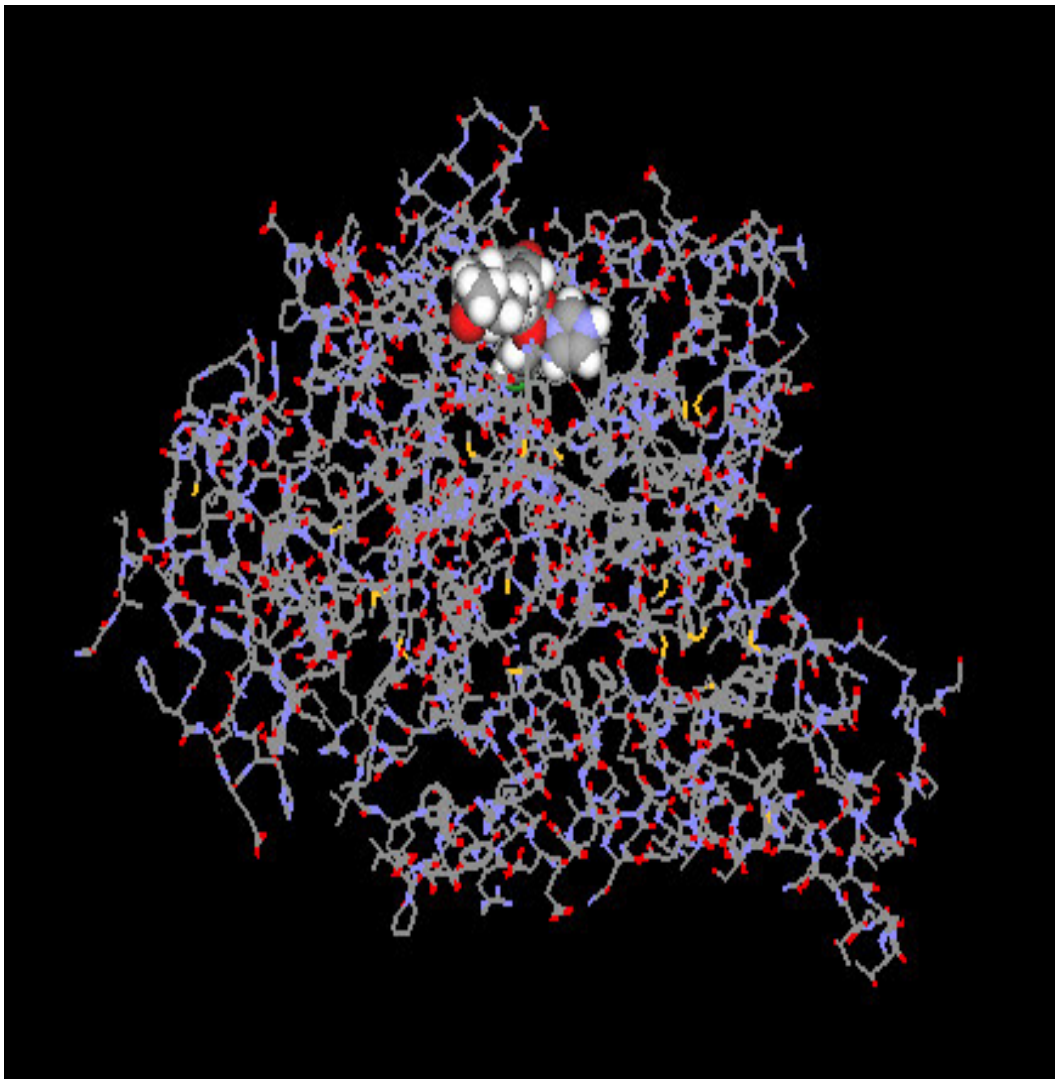


Figure 1. Demetylase enzyme bound with ketoconazole.

ted with azole toxicity in mycotic therapies.

From this docking study, it has been found that the binding sites is hydrophobic and prefer ligands that are hydrophobic to improve contacts. All ligands that were hydrophilic had low scoring function and could not make it to the hit list. Ligands with best score such as ligand 236 is a derivative of testosterone, itself a substrate of other cytochrome enzymes. This can be explained by the fact that the natural ligand, lanosterol, which is typically hydrophobic, formed only one hydrogen bond with 433 Met residues in a region inside the binding pocket. It has been found that mutation at 78 Phe resulted in ligands loosing affinity to the enzyme (Podust, et al., 2001) but none of our selected ligands failed to bind this particular residue which suggests that they may have activity against the mycobacterial bacillus.

Azoles which have shown activity against the enzyme and the whole organism *in vitro* were also docked and the results are included in the table and results are fairly consistent and in agreement with other past studies even though in different isozyme. It can be said that these azoles have modest binding free energy and all three had the binding energy higher than our ligands.

After the docking process, optimization by combining fragments of different drugs that were binding different parts of the pocket. Several permutation and combinations resulted into compounds with significantly lower binding energy and one of them is SPSM1. It is a big molecule and it needs to be further optimized to introduce drug like characteristics. The tris (dichlorophenyl) moiety binds deeper and interacts with the heme while the naphthalyl moiety binds the enzyme lid (Figure 2).

In this study, it was found that the aromatic moie-

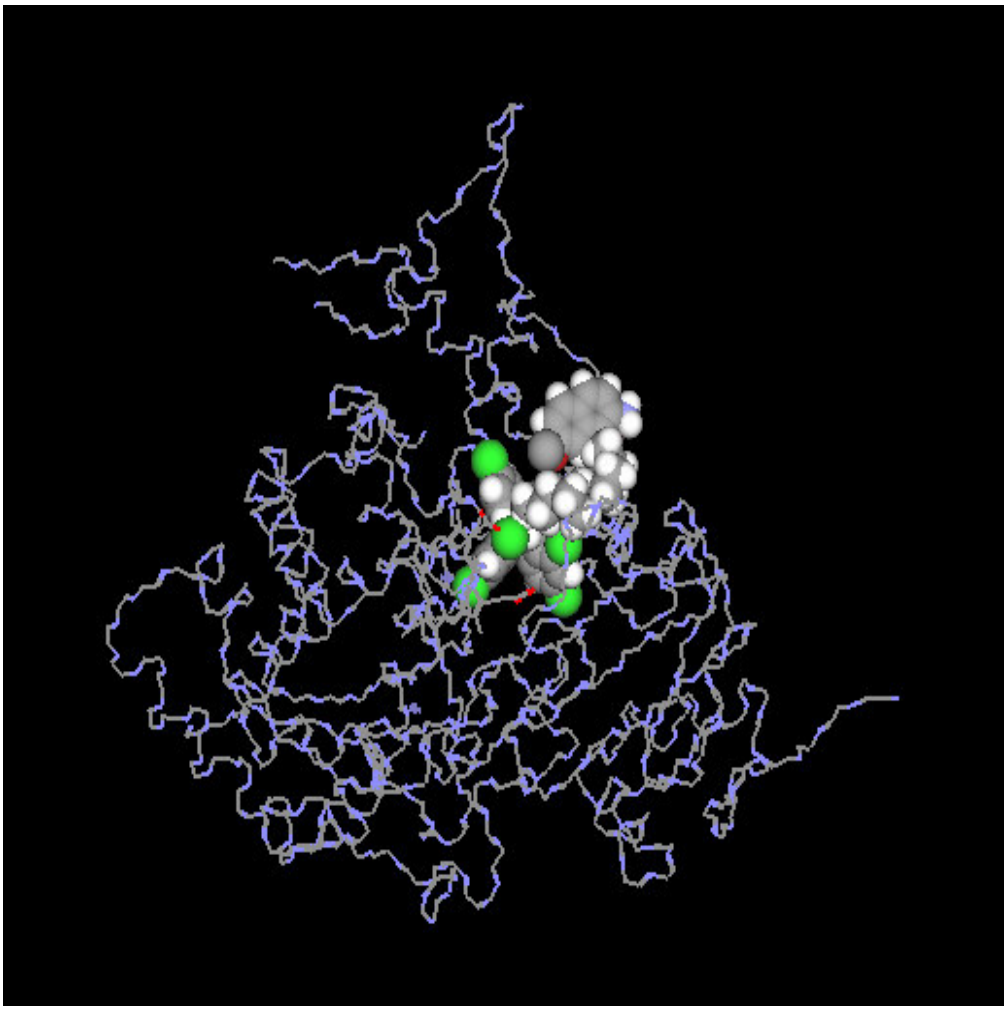


Figure 2. Demethylase enzyme bound with SPSM1.

ties of all drugs where aligning to phenylalanine and tyrosine residues that lines the hydrophobic part of the binding pocket which is itself generally hydrophobic.

After docking, one report published in 2006 indicated that some compounds have been screened for their antitubercular activities at TAACF, Southern Research Institute (www.taacf.org). It should be noted that IC90 results correlated well with the binding free energy as azoles with lower energy and had higher potency. Different from azoles, ligand 241 (Glafenin) showed no potency and this might have arisen from its hydrophilic nature which lower its up-take and capacity to reach the target enzyme because the mycobacterial cell wall is hydrophobic. Ligand 235 (clomiphene) is less potent than azoles against the *M. tuberculosis* even though it had higher binding free energy and it is considered that it is due to its over-reliance on its hydrophobic moiety alone without making any hydrogen bonds to demethylase enzyme (Table 1).

Conclusion

Over 10,000 library compounds have been screened against 14α-lanosterol demethylase of *M. tuberculosis*. Ten hits have been obtained that bind the enzyme at lower energy which is an indicator of their capacity to kill the *M. tuberculosis*. They bind at lower energy than azoles which are known to act against the enzyme and the *M. tuberculosis*. Further optimization resulted into new leads that bind the enzyme at even lower energies. Some ligands that have been identified in this study were earlier tested for their antitubercular activities by the Southern Research Institute in 2006 and have shown potency which correlates well with binding free energies and efforts are underway to test the remaining untested compounds. Also, the work is ongoing to synthesize and test the designed compound SPSM1. It can be concluded that this work has provided an opportunity for further development that may result in novel agents against the disease.

Table 1. Compounds, their heme interaction tendencies, hydrogen bonding properties, binding affinities and their IC_{90}.

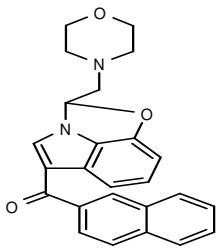
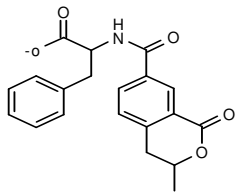
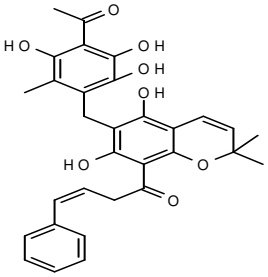
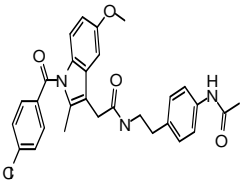
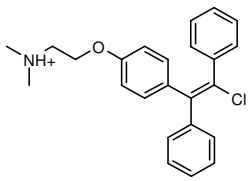
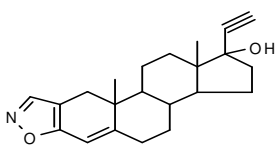
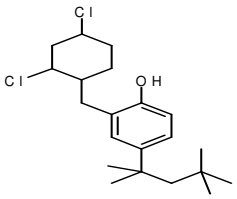
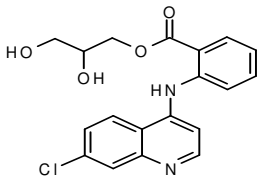
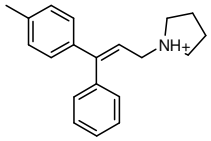
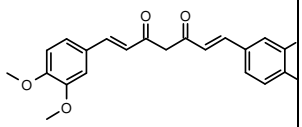
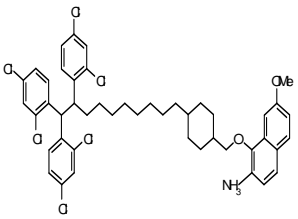
Best structural pose	Amino acids	Hydrogen bond lengths and residues	Binding energy (Kcal/mol)	IC_{90} (µg/mL) H37Rv (as determined by TAAF, Southern Research Institute
Ligand 17 WIN 55, 212-2 	No interaction with the heme	101 HIS: 2.85Å 252 SER: 2.69 Å	-13.8009	Not yet invesitigated
Ligand 190 ochratoxin 	No interaction with heme	252 SER: 2.92 Å, 256 ALA:2.427 Å, 259 HIS: 2.853 Å and 2.3026 Å	-13.3285	Not yet investigated
Ligand 193 Rottlerin 	Interacts with heme	252 SER: 2.88 Å 256 ALA: 2.93 Å 256 ALA: 2.439 Å	-13.5301	Not yet investigated
Ligand 221 n-(acetamidophenyl-) indomethacinomide 	Interacts with heme.	252 SER: 2.908 Å 322 ILE: 2.8962 Å 323 ILE: 2.5193 Å 323 ILE 2.3397 Å	-13.7611	Not yet investigated
Ligand 235 clomiphene 	Interacts with heme	NO H-BONDS	-13.3404	5.789
Ligand 236 danazol 	No interaction with heme	433 MET: 2.638 Å 252 SER: 2.390 Å	-14.8985	Not yet investigated

Table 1. contd.

Ligand 237 	No interaction with heme	259 HIS: 2.993 Å	-13.9946	Not yet investigated
Ligand 241 Glafenine 	Interacts with heme.	252 SER: 2.908 Å 322 ILE: 2.896 Å 323 ILE: 2.5193 Å 323 ILE: 2.3397 Å	-13.7602	>20
Ligand 242 triprolidine 	Interacts with heme	NO H-BONDS	-13.6526	>20
Ligand 246 curcumin 	No interaction with heme.	NO H-BONDS	-13.4784	>20
SPSM1 	Interacts with heme		-19.979	Not yet investigated
ketoconazole		393 ARG: 2.3084 Å 390 GLY: 2.6805 Å	-11.5148	2.563
Miconazole	.	NO H-BOND	-12.2087	1.594
Fluconazole		252 SER: 2.998 Å 101 HIS: 2.9252 Å 72 GLN 2.999 Å	-8.95039	

REFERENCES

Aziz MA, Wright A, Laszlo A, De Muynck A, Portaels F, Van Deun A, Wells C, Nunn P, Blanc L, Raviglione M (2006). Epidemiology of antituberculosis drug resistance (the Global Project on Anti-tuberculosis Drug Resistance Surveillance): an updated analysis. Lancet, 368: 2142-2154.

Girón RM, Máiz L, Barrio I, Martínez MT, Salcedo A, Prados C (2008). Nontuberculous Mycobacterial Infection in Patients With Cystic Fibrosis: A Multicenter Prevalence Study. Archivos de Bronconeumología (English Edition), 44: 679-684.

Guardiola-Diaz HM, Foster LA, Mushrush D, Vaz ADN (2001). Azole-antifungal binding to a novel cytochrome P450 from Mycobacterium tuberculosis: implications for treatment of tuberculosis. Biochemical Pharmacol. 61: 1463-1470.

Hosaka N, Kameko M, Nishimura H, Hosaka S (2006). Prevalence of tuberculosis in small pulmonary nodules obtained by video-assisted thoracoscopic surgery. Respiratory Med. 100: 238-243.

Jackson CJ, Lamb DC, Marczylo TH, Parker JE, Manning NL, Kelly DE, Kelly SL (2003). Conservation and cloning of CYP51: a sterol 14[alpha]-demethylase from Myco-bacterium smegmatis. Biochemical Biophysical Res. Communications, 301: 558-

563.
Madariaga MG, Lalloo UG, Swindells S (2008). Extensively Drug-resistant Tuberculosis. Am. J. Med. 121: 835-844.
Micheal P, Pietila PKV, Bharati S, Nancyu L, Wengenack Sreekumar R, Charles F, Thomas JR (2006). Cloning and characterization of CYP51 from Mycobacterium avium. Am. J. Respiratory Cell Mol. Biol. 35: 236-242.
Mishra P, Hansen EH, Sabroe S, Kafle K (2006). Adherence is associated with the quality of professional-patient interact-tion in Directly Observed Treatment Short-course, DOTS. Patient Education and Counseling, 63: 29-37.
Podust L, Poulos TL, Waterman MR (2001). Crystal structure of cytochrome P450 14 alpha - sterol demythylase (CYP51) from Mycobacterium tuberculosis in complex with azole inhibitors. Proc. Natl. Acad. Sci. USA, 13(98): 3068-3073.
Podust LM, Stojan J, Poulos TL, Waterman MR (2001). Substrate recognition sites in 14[alpha]-sterol demethylase from comparative analysis of amino acid sequences and X-ray structure of Mycobacterium tuberculosis CYP51. J. Inorganic Biochem. 87: 227-235.
Wandwalo E, Makundi E, Hasler T, Morkve O (2006). Acceptability of community and health facility-based directly observed treatment of tuberculosis in Tanzanian urban setting. Health Policy, 78: 284-294.