Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(2):169-178

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Design, synthesis and screening of inhibitor of Cytochrome P450 1B1 (CYP1B1)

Mitesh Hood*, David Adams and Philip Roberts

School of Pharmacy and Pharmaceutical Sciences, University of central Lancashire, Preston, United Kingdom

---

ABSTRACT

Cytochrome P450 1B1 is considered to be the prime reason of the cancer cells becoming resistance towards anti-cancer drugs. It is involved in metabolism of anti-neoplastic agents as well as bioactivation of various polycyclic aromatic hydrocarbon carcinogens. Therefore, it is of vital importance to find a selective inhibitor of CYP1B1 enzyme, which might aid in the anticancer therapies in near future. The compound 2,3,4-trimethoxy-4'-methylthiostibene is a well-known inhibitor of CYP1B1. This was chosen as a lead compound, from which an analogue that interacts with CYP1B1 was evaluated by the process of virtual screening. The resultant compound, thus selected, was modified and later synthesized. The structure and identity of the synthesized product was confirmed by thin nuclear magnetic resonance spectrometry (NMR) and mass spectrometry (MS). Later the synthesized product was subjected to 7- Ethoxyresorufin O-deethylation (EROD) assay in order to test its biological activity. Form the experiments carried along, the synthesized analogue was confirmed for its inhibitory activity against CYP1B1 enzyme.

Keywords: CYP1B1, EROD, Anti-cancer, Cytochrome P450, Ehoxyresorufin, Enzymes, Cancer.

INTRODUCTION

I. Cytochrome P450 Enzymes:

The cytochrome P450 (CYP450) enzymes are a group of heme-containing enzymes which take part in the metabolism of many drugs, steroids and carcinogens. This ubiquitous family consists of more than fifty enzymes which catalyse a large diversity of transformations involved in phase I metabolism of various xenobitoics including anti-neoplastic drugs. In terms of functionality these enzymes can be broadly classified into two classes viz. those that have a role in endogenous molecule metabolism (e. g. hormones) and those which carry out exogenous molecule biotransformation (e. g. dugs).

While most of the CYP450 enzymes are found in the liver, CYP1B1 is an exception as it is commonly found in the extra hepatic tissues and cells. The presence of CYP1B1 in ovary, testis, adrenal gland (steroidogeinc tissues) and prostate, uterus and breast (steroid-responsive tissues) is noteworthy [1]. From immunohistochemistry studies it has been shown that this protein plays an active role in a wide range of cancers including cancers of the esophagus, lymph, brain, including also lung and breast cancer where this enzyme is over expressed [2]. It is also involved in the metabolic activation of benzo(a)pyrene, 5-methylchrysene, dibenzo(a,l) pyrene anthracene and various other polycyclic aromatic hydrocarbon carcinogens [3]. CYP1B1 is also found to have a significant role in the metabolism of 17β-estradiol and converts it into 4-hydroxyestradiol, a key intermediate that leads to a quinone DNA-adduct responsible for estrogen related carcinogenesis [4]. It is because of these characteristics that CYP1B1 is of great

research interest .The CYP1B1 enzyme has been shown to be present in almost all healthy extra hepatic tissues. However, allelic discrepancies in CYP1B1 are thought to account for the frequencies of quite a few categories of cancer [5]. The over expression of CYP1B1 in tumour cells also affect the activities of anti-neoplastic agents. Mcfadyen in 2001 showed the over expression of CYP1B1 in malignant cells and this fact may account for the reason cancer cells become resistance towards anti-cancer drugs [6]. Some of the drugs which are affected are taxanes, cyclophosphamides amongst others [7]. Thus, inhibition of CYP1B1 maybe of importance in overcoming the malignant cells resistance to treatment.

CYP1B1 is a member of the CYP1 family which consists, in addition to CYP1B1, CYP1A1 and CYP1A2. The x-ray structures of all three enzymes are available. It has been shown that CYP1B1 has low sequence identity with both CYP1A1 (38%) and CYP1A2 (37%). However, despite this low sequence homology, all three enzymes share substrates and inhibitors, although there are functional similarities as well as differences. It appears that CYP1B1 and CYP1A1 have a similar substrate and inhibitor profile, possibly accounting for a lack of selective CYP1B1 inhibitors. Hence, need for a further innovative approach to identify such compounds [9] [10].

II. Inhibitors of CYP1B1:

CYP1B1 is over expressed in many tumor types relative to normal tissues and evidence for its role in tumorigenesis is supported not only by its increased expression but also by its ability to activate several classes of carcinogens. An obvious strategy for chemoprevention would therefore be the inhibition of CYP1B1.

Resveratrol (3',4',5-trans-trihydroxystilbene), is a naturally occurring phytoalexin which shows a broad spectrum of biological activity, including anti-tumor, anti-inflammatory, anti-oxidative effects. The compound is chiefly accepted as a cancer chemopreventive as it is able to block various stages involved in process of carcinogenesis. Inhibition of DNA polymerase, cyclooxygenase, ribonucleotide reducatase and kinases are some of the main molecular targets of inhibition of resveratrol [8]. Ragione in 1998 stated that resveratrol leads to S/G2 transition phase arrest in the cell cycle, and thus is able to induce apoptosis in a wide range of cancer cell lines like, prostate, breast, leukemia and etc. [11] [12] [13] [14] [15].

The cytochrome P450 catalyzed hydroxylation of resveratrol and thus the formation of piceatannol, denotes a fact that can b transformed into a recognized chemopreventive/ anti-neoplastic active compound by CYP1B1 [16]. In contrast to the abundant studies carried on resveratrol, a little amount of research worked has been carried out on the other hydroxylated stilbenes. However, it has been confirmed that the analogues possessing 3,4,5- trihydroxy-trans-stilbene, 3,4,4' - trihydroxy-trans-stilbene , 3,4-dihydroxy-trans-stilebenes and other 3,4-dihydroxy groups show increased amount of antioxidant and apoptotic activity than reseveratrol [17].

III. Virtual Screening and Drug Discovery:

The term virtual screening describes the use of computational algorithms and models for the identification of novel bioactive molecules. The method provides valuable starting points for drug discovery as a complement to high-throughput screening [18]. Virtual screening according to Sheridan, can be classified into four types viz. substructure searching , structure similarity searching, docking and QSAR. The method undertaken in this project was the searching of a virtual three dimensional structural database for compounds of 3D structural similarity to a chosen lead compound [19]. The method is based on the principle that compounds having a similar structure tend to have similar biological activities. The method has been used since the 1980's and is still widely used today, although the process has become more refined and dependable in terms of hit rates [20] [21].

Substructure and 3D pharmacophore searching involve the specification of a precise query, which is then used to search a database in order to identify molecules for screening. In such an approach, either a molecule matches the query or it does not. Similarity searching offers a complementary approach, in that the query is typically an entire molecule. This query molecule is compared to all molecules in the database and a similarity coefficient is calculated. The top scoring database molecules (based on the similarity coefficient) are the 'hits' from the search with the expectation that the 'hits' obtained would show similar pharmacological activity as the query molecule. In order to compute the similarity between two molecules, a set of descriptors needs to be defined in order to compute the similarity coefficient. These molecular descriptors can be those easily calculated from the molecular formula, such as the molecular weight. Some may be determined experimentally, such as the partition coefficient, with which a calculated value can be compared, while others can be purely computational such as a fingerprint. Other descriptors

may be time consuming to calculate such as those derived from quantum mechanics, for example, the molecular electrostatic potential [22] [23].

A recent approach has been to calculate a similarity coefficient based on the molecular descriptors of shape and electrostatic potential (ShaEP) as described by [24]. In this approach the molecular similarity is detected through the calculation of the electrostatic potentials and rigid body superimposition where the superimposition is scored by the degree of overlap and then expressed as the Hodgkin similarity index.

The process of superimposing two rigid molecular structures and thus computation of the similarity co-efficient based on the shape and electrostatic potential can be easily carried out by a virtual tool named as ShaEP. This virtual tool gives a very simple and efficient solution to the conformational flexibility problem by assessing the multiple structures that are present in the database in a sequential manner [24]. The similarity index, thus calculated, is useful in screening of the potential analogues which are in structural resemblance with the ligand.

The process of ligand protein docking and the prediction regarding binding of a compound/analogue to a receptor can be easily achieved by a flexible program named Autodock. The program enables calculation of a plot of protein ligand interactions of the binding site with the docking ligand. This Autodock is the engine behind a program named PyRX; a molecular docking and visualizing software. The dual advantage of this program enables the process of virtual screening from the preparation of protein and ligand to the result analysis. This makes the process of virtual screening and docking much easier simpler and faster.

IV. 7- Ethoxyresorufin O-deethylation (EROD) Assay:

The EROD assay indicates the amount of CYP1B1 mediated deethylation of 7-ethoxyresorufin (7-ER) to resorufin. In this assay, the catalytic potential/activity of CYP1B1 is used for its detection. The amount of 7-ER that gets converted to resorufin, thus becomes an indicator of the presence of CYP1B1. The assay involves the mixing of 7-ER and NADPH with the sample and later measuring fluorometrically the resorufin formed [25].

Generally, microsomes are usually used in EROD assay. However, microsomes were unsuitable for this research project assay as it contains CYP1A1 in detectable quantities. Thus, selective inhibition of CYP1B1would not be measured. Hence this assay was modified. Instead of microsomes, CYP1B1 over expressed bactosomes were used. These bactosomes are human CYP1B1 and CYP-reductase co-expressed in Escherichia coli. Thus when 7-ER is added to the bactosomes along with NADPH, in the presence of CYP1B1 it is converted into resorufin. This is later measured by the fluorescence produced by resorufin, by fluorimetric analysis.

1. *Methodology/ Virtual Screening*:

To begin, Sketchel program was used. Sketchel is chemical molecule sketching tool with data entry application. The program allows the designing of two dimensional structures, storage in a molecular spreadsheet and most importantly allowing to export the saved file to .sdf format.

Initially, the programme was used in order to draw a two dimensional figure of the lead compound. This structure was later stored in the sketchel sheet, which was further exported as a structure data (.sdf) file.

In order to convert the two dimensional structure to 3D, the program named OpenBabel was used. The programme can be used easily for the conversion, analysis and storage of the data [23]. During this process the hydrogen atoms were integrated to the compound.

Minimization and addition of MMFF94 charges to the molecule was done by using balloon and thus a mol2 file was created. A series of low energy conformers were obtained by this process from which the lowest energy conformer was chosen for the virtual screening.

The resulting 3D mol2 file was keyed in to a program named ShaEp, a basic molecular tool for handling the superimposition and similarity analysis [24]. A similarity and superimposition analysis of the molecule was carried out which was based on the electrostatic charge and the shape of the actual molecule. In order to achieve the goal of getting a preliminary idea of both, the shape and the electrostatic potential, the process was carried based on the subset of the ZINC database. Thus, the intention of superimposing the 3D generated lead compound with other related 3D structures and calculation of the similarity index was achieved. The program commands were set in such

a fashion that only 50% and above similarity with respect to the lead compound structure were isolated. These set of compounds were later stored as .sdf file. After 6 hours, 250 potential ligands were generated from the ZINC library, which contained 330,000 compound structures. These 250 compounds (hits), thus obtained were again entered in the OpenBabel in order to remove hydrogen atoms and thus converting the structure to 2 dimensional figure for ease of visualization.

The next step was to retrieve these structures in a readable format, which was achieved through SDF toolbox and a .sdf file containing all the structure was created and saved. Finally, the .sdf file was exported in an excel spreadsheet using molecular database/spreadsheet.

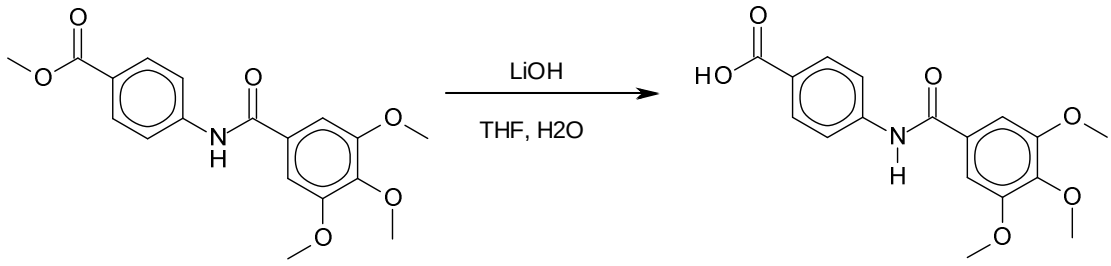
The 250 compound structures, thus obtained were analyzed carefully in all its preliminary perspective which included the probability and ease of synthesis process, active replacements as well as their pharmacological action and potential. From this, the most suitable compounds were selected and a simple and specific experiments plan was framed.

EXPERIMENTAL SECTION

I. Synthesis Of 4-[(3,4,5-trimethoxy benzoyl)amino]benzoic acid (Compound M1)

Methyl 4-[(3,4,5-trimethoxybenzoyl)amino]benzoate (1.23 g; 0.005 moles)and lithium hydroxide (0.24 g, 0.010 moles), were combined in tetrahydrofuran (THF) (10 ml) and water (2.2ml) was added drop wise until a solution was formed.

The mixture thus obtained was stirred at room temperature for 16 h. The THF was then removed in *vacuo* and the residue was acidified using 10% HCl until pH 4 was obtained. The resultant precipitate was filtered to afford methyl 4-[(3, 4, 5-trimethoxy benzoyl) amino] benzoic acid, which was recrystallized with methanol and water. NMR (DMSO-d^6) δ: 12.79 (1H, brs) 10.40 (1H, brs) 7.92 (4H, q) 7.28 (2H, s) 3.87 (6H, s) 3.73 (3H, s).



methyl 4-[(3,4,5-trimethoxybenzoyl)amino]benzoate

Compound M1

II. Bioassay: De-alkylation of Ethoxyresorufin Assay

O-deethylation of ethoxyresorufin (EROD) assay was chosen in order to analyse the biological activity of the compounds. In order to measure the activity of CYP1B1, the production of resorufin from the ethoxyresorufin was measured. The potential analogues of CYP1B1, obtained from the above experiments, were dissolved in acetonitrile and later arranged in the serial concentration of 100µm, 10µm and 1µm. While carrying out the assay procedure, 20µl of bactosomes, 20µl of ethoxyresorufin, 120µlof phosphate buffer and 20µl of NADPH was added in 96 well plates. This mixture, thus prepared was run against the control (mixture without drug) under fluorescence under 530nm excitation and 590nm emission. A separate run test run for acetonitrile was carried along during the assay to analyze the vehicle toxicity.

RESULTS

I. Virtual screening and synthesis strategies:

Based on proposed modifications to the lead compound, eight analogues with a satisfactory practical yield were prepared.

II. Identification/Analysis of the compounds:

In order to have a complete affirmation regarding the identification of the synthesized analogue, all the compound was analysed using mass spectral analysis. Once the identification of each potential analogue was confirmed, they were evaluated for biological activity.

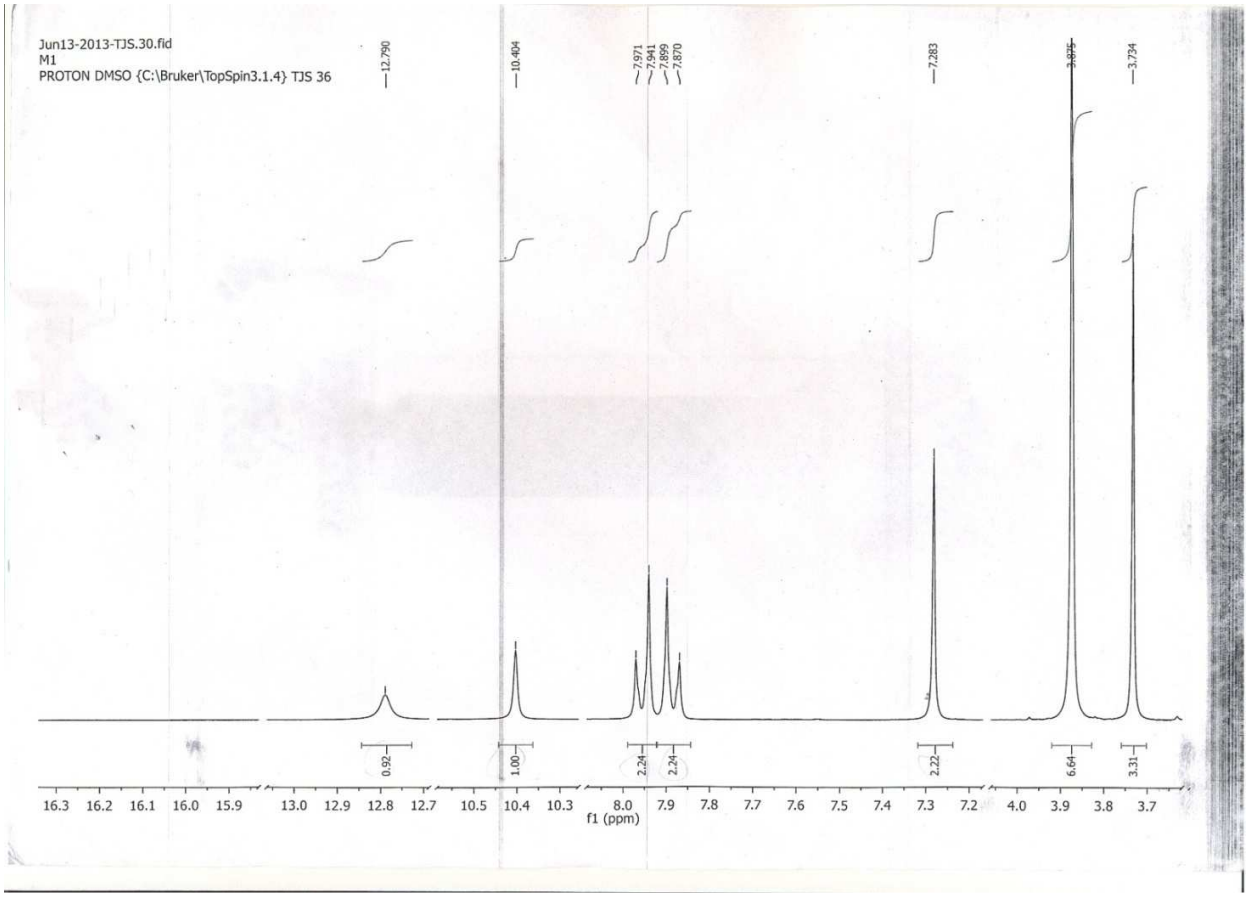


Figure 1: NMR (DMSO-d6) δ: 12.79 (1H, brs) 10.40 (1H, brs) 7.92 (4H, q) 7.28 (2H, s) 3.87 (6H, s) 3.73 (3H, s)

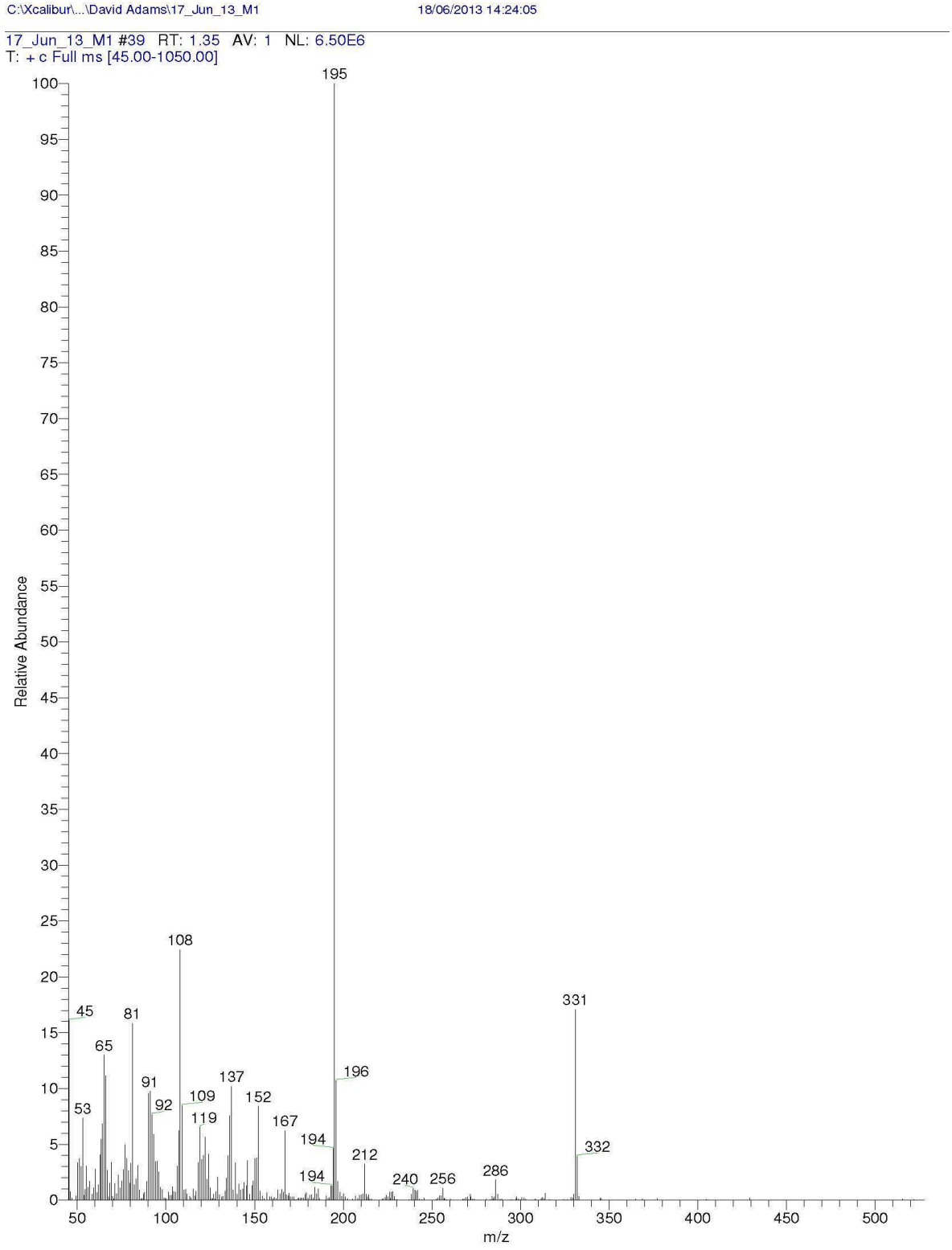


Figure 2: Mass Spectrometry of Compound M1: MS (amu) 331(M^+); 195 ($C_{10}H_{11}O_4$)

III. 7-ethoxyresorufin-O-deethylation (EROD) assay:

Influence of CYP1B1 activity, was carried and tested along by measuring the resorufin production from dealkylation of 7-ethoxyresorufin. Initially, the activity of ethoxyresorufin was determined. The process of analyzing the compounds was done only after the whole assay was carried out successfully and thus the positive results regarding the assay were confirmed.

The results of all the experiments including the identification/analysis as well as biological activity of the compounds are stated as under:

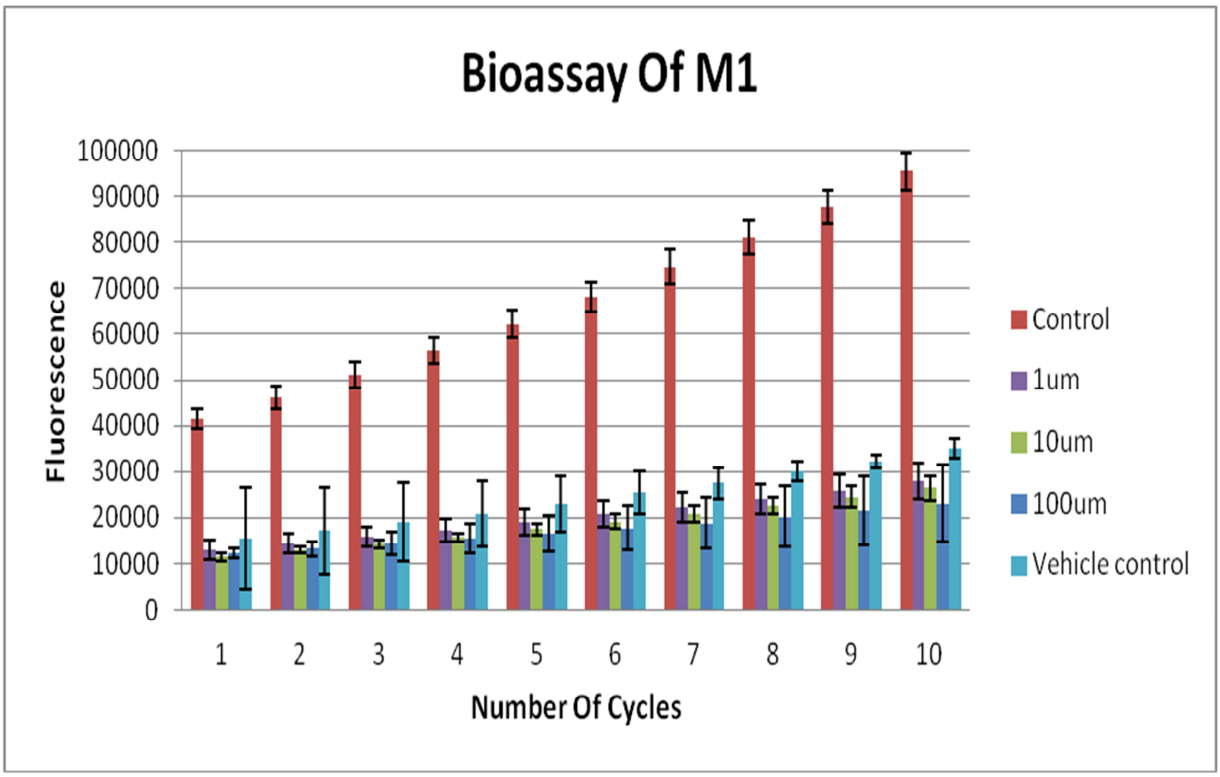


Figure 3: EROD Bioassay of Compound M1

The graph displays the EROD bioassay results of the compound M1. The X-axis represents the number of cycles all through the assay. The time interval required for every cycle was 30 seconds. Y-axis represents the fluorescence values for resorufin, noted for every cycle. Control represents the expression of CYP1B1 (without any compound), followed by the ascending concentrations of the drug and vehicle control (acetonitrile).

DISCUSSION

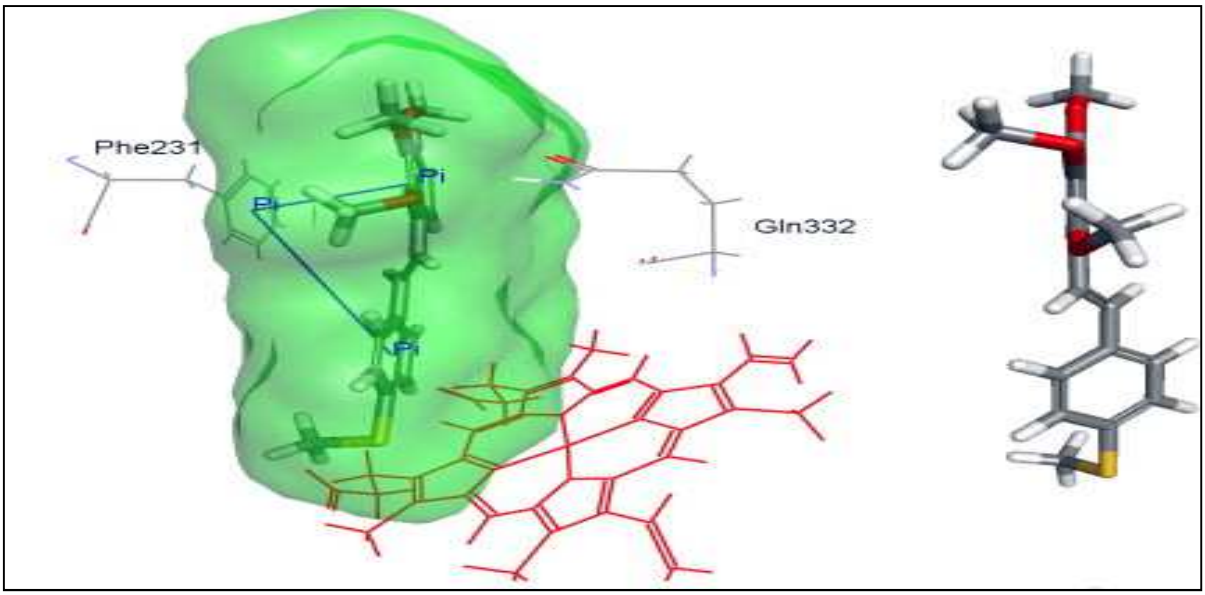


Figure 3: Docked 2,3,4-trimethoxy-4'-methylthio-trans-stilbene x-ray crystal structure of CYP1B1. Source: [26]

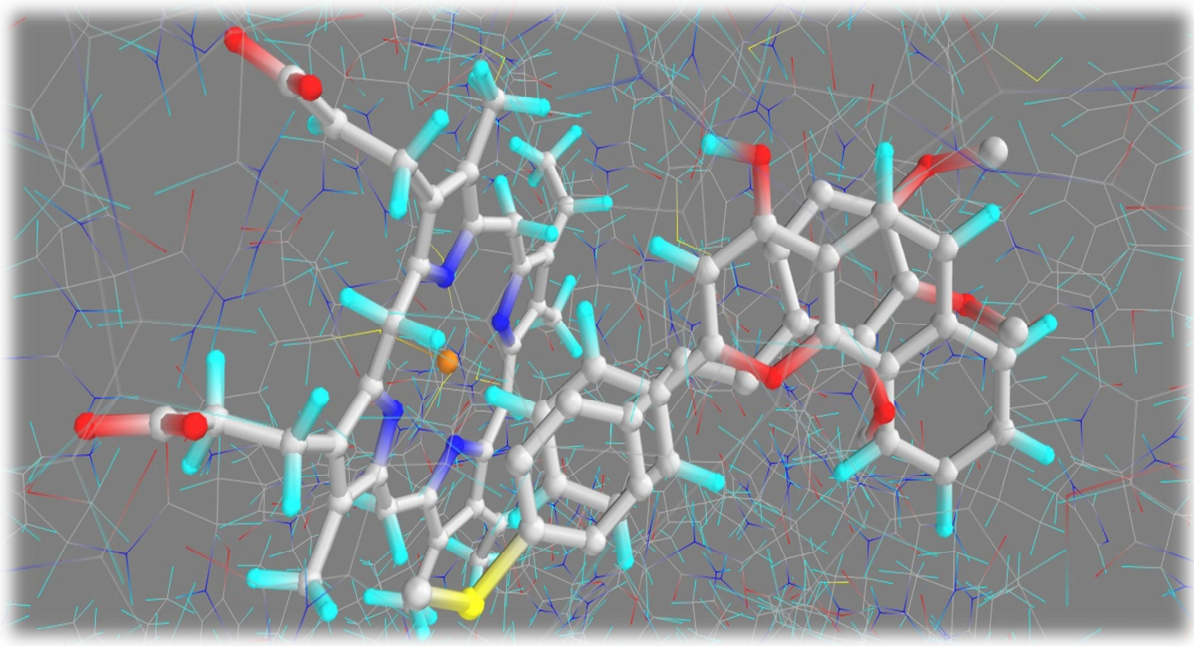


Figure 4: Docked 2,3,4-trimethoxy-4'-methylthio-trans-stilbene superimposed X-ray crystal structure of CYP1B1 containing α-napthoflavone (PyRx)

The postulated binding interaction of 2,3,4-trimethoxy-4'-methylthio-trans-stilbene with CYP1B1 enzyme is shown in the figure 3. Analysis of the results given by Mikstaca (2012) showed that the B-ring containing the 4'-methylthio substituent is directed towards the heme. Also π-π stacking of the two rings with Phe 231 is found. In addition, a possible H-bond exists between Gln332 and the 2-methoxy substituents in the A ring [26].

In this work, docking of 2,3,4-trimethoxy-4'-methylthio-trans-stilbene with CYP1B1 (downloaded from RSC PDB as 3PMO) using Autodock under PyRx also showed that the sulfur containing aromatic ring B was turned towards heme because of the co-ordination between sulfur atom and heme.
The pose identified by Mikstaca was confirmed in this work. Together with virtual screening and the docking results structural modifications of 2,3,4-trimethoxy-4'-methylthio-trans-stilbene were considered.

Compound M1:

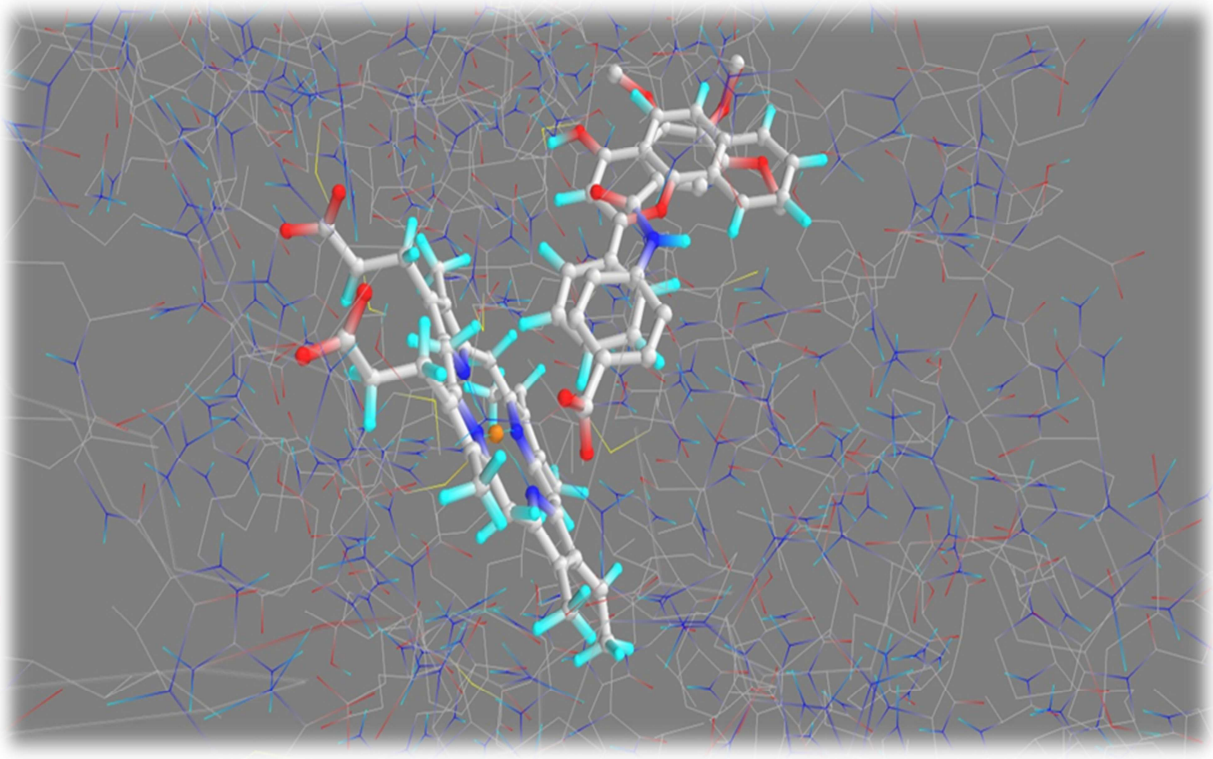


Figure 5: Docked Analogue M1 superimposed X-ray crystal structure of CYP1B1 containing α-napthoflavone

The modification considered in 2,3,4-trimethoxy-4'-methylthio-trans-stilbene were:

I. The replacement of the sulfur containing group with a carboxylic acid group as a heme coordinating group. Similar modifications, i.e. replacement of a sulfur containing group by a carboxylic acid, were made in the development of the ACE inhibitors for the treatment of implementation where a sulfhydryl coordinating group as in captopril was replaced by an acid group as in enalaprilat.

II. The replacement of olefinic linker with a bioisosteric amide groupings for ease of synthesis and improved drug-like characteristics (calculated Log P 1.8 vs 3.7)
Docking of M1 as shown in the figure 5 using Autodock in PyRx gave a similar pose to the 2,3,4-trimethoxy-4'-methylthio-trans-stilbene with CYP1B1. It can be postulated that the acid group interacts with the heme group in a similar manner.

The compound M1 was synthesized in two step process from the combining of Methyl 4-[(3,4,5-trimethoxybenzoyl)amino]benzoate and THF followed by basic hydrolysis. The reaction progress in each case was

followed by thin layer chromatography (TLC) and worked up when TLC indicated that the reaction was completed. After isolation and purity check by TLC the product was aalysed by NMR and MS.A mass spectrum showing 331(M^+) and a margin fragment at 195 ($C_{10}H_{11}O_4$) was obtained.

The fact that the product was a potent CYP1B1 inhibitor was confirmed by EROD bioassay (figure 3). The inhibition of the CYP1B1 in the bactosomes was clearly visible from the assay.

CONCLUSION

The figure 1 and figure 2 shows the NMR and the mass spectrum results of M1 analogue respectively, thus confirming its structure. The mass spectrum results showed the parent peak at 331 (M^+) and a base peak of 195.
The EROD assay, displayed the efficient inhibition of CYP1B1. The inhibitory activity of this compound was found to be dose dependent. Thus, It can be said that the analogue M1 is a potential CYP1B1 inhibitor.

REFERENCES

[1] K.N. Degtyarenko (**1995**). *Protein Engineering*. 8 (8), 737-747.
[2] C Young-Jin, L Sang-Kwang, and K Mie Young. (**2005**). *Drug Metabolism And Disposition*. 33 (1), 1771-1776.
[3] GI Murray, MC Taylor, MC McFadyen, McKay JA, Greenlee WF, Burke MD, Melvin WT. (**1997**). *Cancer Res*. 57 (1), 3026-3031.
[4] B Rochat, JM Morsman, GI Murray, WD Figg, HL McLeod (**2002**). *J Pharmacol Exp Ther*. **2001**. 296 (2), 537-541.
[5] S Bandiera, S Weidlich, V Harth, P Broede, Ko Y, T Friedberg . (**2005**). *Mol Pharmacol*. . 67 (2), 435-443.
[6] MC McFadyen, WT Melvin, GI Murray. (**2004**). Cytochrome P450 CYP1B1 activity in renal cell carcinoma.. . 91 (5), 966-71.
[7] T Shimada, Y Fujii-Kuriyama. (**2004**). *Cancer Science*. 95 (1), 1-6.
[8] Audrey Laroche-Clary1, Valerie Le Morvan1, Takao Yamori2, and Jacques Robert1. (**2010**). *Molecular Cancer Therapeutics* . 9 (1), 3315-3321.
[9] Joanna Trubicka1, Ewa Grabowska-Kłujszo1, Janina Suchy1, Bartłomiej Masojć1, Pablo Serrano-Fernandez1, Grzegorz Kurzawski1, Cezary Cybulski1, Bohdan Górski1, Tomasz Huzarski1, Tomasz Byrski1, Jacek Gr. (**2010**). *BMC Cancer*. 10 (1), 420.
[10] NJ Walker, JA Gastel, LT Costa, GC Clark, GW Lucier, TR Sutter. (**1995**). *Carcinogenesis*. 16 (6), 1319-1327.
[11] Damianaki A, Bakogeorgou E, Kampa M, Notas G, Hatzoglou A, Panagiotou S, Gemetzi C, Kouroumalis E, Martin PM, Castanas E.. (**2000**). *J Cell Biochem*. 78 (3), 429-441.
[12] SC Gautam, Xu YX, M Dumaguin, N Janakiraman, RA Chapman. (**2000**). *Bone Marrow Transplant.*. 25 (6), 639-645.
[13] E Pozo-Guisado, A Alvarez-Barrientos, S Mulero-Navarro, B Santiago-Josefat, PM Fernandez-Salguero. (**2002**). *Biochem Pharmacol*.. 64 (9), 1375-1386.
[14] Y Schneider, F Vincent, B Duranton, L Badolo, F Gossé, C Bergmann, N Seiler, F Raul. (**2000**). *Cancer Letters*. 158 (1), 85-91.
[15] HB Zhou, Y Yan, YN Sun, JR Zhu. (**2003**). *World J Gastroenterol*.. 9 (3), 408-411.
[16] GA Potter, LH Patterson, E Wanogho, PJ Perry, PC Butler, T Ijaz, KC Ruparelia, TH Lamb , PB Farmer, LA Stanley, MD Burke. (**2002**). *Br J Cancer*.. 86 (5), 774-778.
[17] LM Hung, JK Chen, RS Lee, HC Liang, MJ Su. (**2001**). *Free Radic Biol Med.* . 30 (8), 877-883.
[18] Y Tanrikul, B Krüger, E Proschak. (**2013**). *Drug Discov Today*. 18 (7-8), 358-364.
[19] AS Reddy, SP Pati, PP Kumar, HN Pradeep, GN Sastry. (**2007**). *Current Protein and Peptide Science*. 8 (4), 329-351.
[20] RP Sheridan, SK Kearsley. (**2002**). *Drug Discov Today*.. 7 (17), 903-911.
[21] S Kortagere, MD Krasowski, S Ekins. (**2009**). *Trends Pharmacol Sci.* . 30 (3), 138-147.
[22] Reed B. Jacob, Tim Andersen, Owen M. McDougal. (**2012**). *PLoS Computational Biology* . 8 (5), 1-5.
[23] O'Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR.. (**2011**). *Journal of Cheminformatics*. 3 (33), 1-14.
[24] MJ Vainio, JS Puranen, MS Johnson. (**2009**). *J. Chem. Inf. Model*. 49 (2), 492-502.
[25] Galia Zamaratskaia and Vladimir Zlabek. (**2009**). *Sensors*. 9 (1), 2134-2147.
[26] R. Mikstackaa, AM. Rimandob, Z. Dutkiewicza, Tomasz Stefańskia, Stanisław Sobiaka. (**2012**). *Bioorganic & Medicinal Chemistry*. 20 (17), 5117–5126.