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Evaluation of antimicrobial potential of synthesized dibromotyrosine analogs using *in vitro* and *in silico* approaches

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ABSTRACT

Many potent compounds rich in therapeutic potential have been isolated from marine sponges but they have failed to make it to the clinical trials or rather failed in the clinical trials because of the extreme cytotoxicity they possess. In the present study we have evaluated the biological potential of synthetic analogues of dibromotyrosine where we assume that the analogues designed and synthesized have retained their potency but lost their cytotoxicity. 2-(3, 5-dibromo-4-hydroxyphenyl) acetamide [analogue 1] and ethyl 2-(3, 5-dibromo-4-hydroxyphenyl) acetate [analogue 2] were tested against bacterial strains DH5α and ER2566 and Candida albicans was taken as fungal counterpart. The antimicrobial activity of both the analogues showed inhibitory effects against both the bacterial strains with ER2566 giving MIC_{50} value of 31.25µg/ml for analogue 1 and 66.19µg/ml for analogue 2 while MIC_{50} value of 21.72µg/ml was obtained for analogue 1 and 17.69µg/ml for analogue 2 against DH5α.The analogues were also antifungal against Candida albicans with MIC_{50} values of 170.50µg/ml for analogue 1 and 145.37µg/ml for analogue 2.Subsequent to the wet lab analysis both the analogues proved to be equally effective when subjected to in silico studies using online softwares and docking tools and showed no toxicity against extracted lymphocytes from rat's blood thereby emphasizing, hopefully correct synthesis of analogues who have probably lost their activity to be cytotoxic.

Keywords: Dibromotyrosine; Aeroplysinin-1; in-silico; Antibacterial; Antifungal; Antiviral.

INTRODUCTION

The enormous resource known for the potential discovery of chemotherapeutic agents lies in the oceans round the world covering almost 70% of the earth's surface containing almost 5,00,000 species of marine organisms. All but two of the 28 major animal phyla are represented in aquatic

environments, with eight being exclusively aquatic, mainly marine. The traditional medicine under its umbrella does not have a significant contribution of marine organisms but the Phoenicians employed a chemical secretion from marine molluscs to produce purple dyes .Isolation of C-nucleotides, spongouridine and spongothymidine from Caribbean sponge, *Cryptotheca crypta* in early 1950s was the first notable discovery of biologically active compounds from marine sources. The association of marine sponges with enormous amount of microorganisms brought them in picture and the microbiologists were so much fascinated by them and named them rightly as 'microbial fermenters' having inherent and untapped potential for therapeutics [1].

A broad arsenal of structurally diverse and pharmacologically active compounds are provided by nature in the name of secondary metabolites derived from marine sources that act as highly effective drugs or lead structures for the development of novel synthetically derived drugs to combat a multitude of diseases. Considerable cost is imposed on the organism producing secondary metabolites involving resources in terms of nutrient and energy [2]

The ecological importance of these compounds derived from respective organisms is clearly evident from the fact that since they are predominantly found in sessile or slow moving marine organisms that lack physical defence structures such as in algae and most marine invertebrates [3] .Despite lacking protecting shells or other physical defence structures these sessile sponges often live exposed and exhibit conspicuous colours which subjects them to face a high risk of predation which is indicated in various studies on the significance of marine natural products acting against predatory or herbivorous fish or other predators [4, 5].

Sponges of the genus *Aplysina* are known for their structurally diverse brominated isoxazoline alkaloids [6-15] which act as potent chemical defence against predators and microorganisms [15-18]. *Aplysina* species occur in the Mediterranean Sea, the Atlantic Ocean, and in the Caribbean Sea [19] where they often contribute to the dominant sponges present. The Mediterranean Sea is home to two *Aplysina* species: *Aplysina aerophoba* which occurs in water depths as low as 1 m [20, 21], and *Aplysina cavernicola* which prefers shaded caves and deeper habitats (40 m or lower) [22, 23].

A. aerophoba typically contains isofistularin-3 [6, 12] and has a highly unstable yellow pigment uranidine which polymerises rapidly when exposed to air and yields a black polymer [6]. This phenomenon is the reason for the name"aerophoba" (fearing air) which refers to the blackening of sponge tissue when exposed to air.

The crude extracts of marine sponges exhibit a high degree of antibacterial activity against terrestrial pathogenic bacteria but low incidence of the same activity against marine bacteria. Despite the discovery of new marine molecules with antibiotic properties, their ubiquity in marine sponges is remarkable. In an early screening study conducted by Burkholder and Ruetzler [24] around 18 of the 31 sponges tested, showed antimicrobial effects and the effect of few of them was very strong against a range of Gram-positive and Gram-negative bacteria.

There is an urgent need of new antifungals in clinical medicine because different kinds of mycoses, especially invasive mycoses, have become an important public health problem as their incidence has increased dramatically in the last decades in relation to AIDS, haematological malignancies, transplant recipients and other immunocompromized individuals. The causative reason for the death of patients who are treated for a malignant disease is fungal infections and to add to this the emerging resistance is also an important problem. [25-31].

Antifungal substances derived from marine sources are not considered promising for clinical applications because of their cytotoxicity.But a review of the marine natural products shows antifungal activity but cytotoxicity was not available for all of them. Therefore the approach should be assessment of whether antifungal activity outweighs the cytotoxic effects followed by rational modifications to improve the therapeutic index for these molecules [32].

The thought which finds the basis of this piece of work deals in the chemical synthesis of marine sponge derived leads in such a manner that the biological potency is retained and cytotoxicity is removed or rather minimized resulting in the overall outweighing of the positive potent effects with the negative side effects.

EXPERIMENTAL SECTION

Material and Methods

Synthetic analogues of marine compound dibromotyrosine

The synthetic analogs of aeroplysinin 1 and dibromoverongiaquinol were received from Dr.Khalid A.El.Sayed , Faculty of Pharmacy, University of Louisiana at Monroe, U.S.A. in powdered form [33]. The analogues were dissolved in chloroform at a stock concentration of 10µg/µl and stored at – 20^0C.

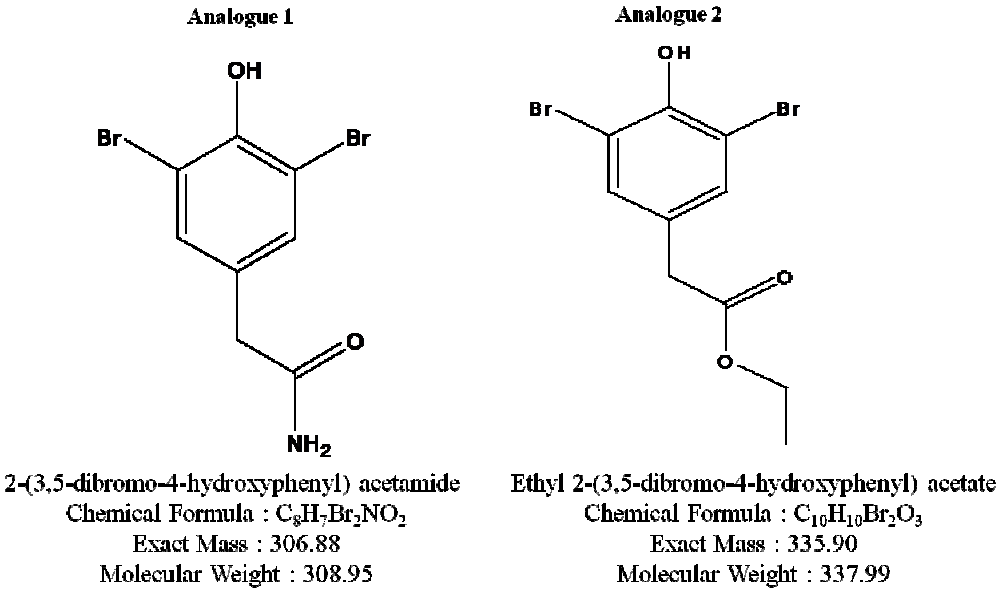

Figure 1: Chemical structures and analysis of analogue 1 & 2

Collection of strains and chemicals

The bacterial strains used in this study were *DH5α* and *ER2566* and the fungal strain used was *Candida albicans* ATCC 90028. The yeast strains were cultured in Yeast Extract Peptone Dextrose 17 (YEPD) broth (BIO101, Vista, Calif.). For agar plates, 2.5% (w/v) bacto agar 18 (Difco, BD Biosciences, NJ) was added to the medium. All strains were stored as frozen stocks with 15% glycerol at -80°C. Before each experiment, cells were freshly revived on YEPD plates from the stock. Commercial grade mixtures of curcuminoids commonly known as curcumin were used in this study.

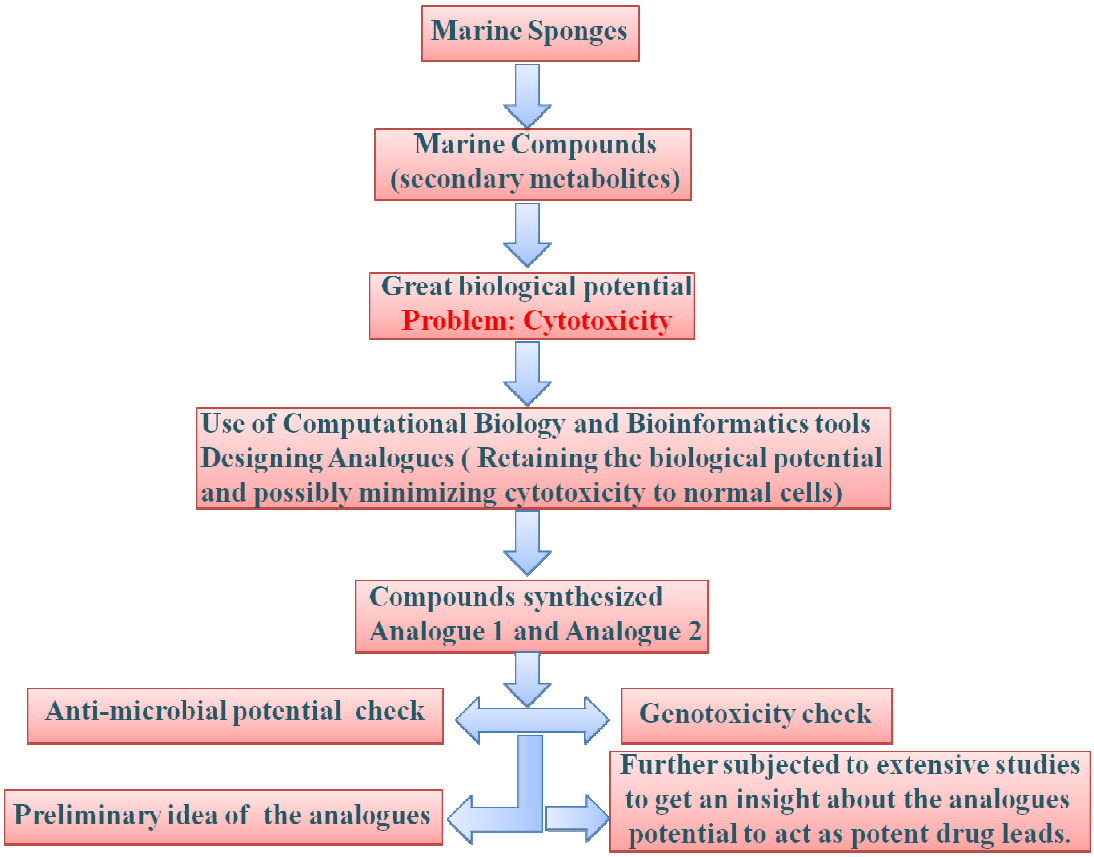

Figure 2: Pictorial representation of the proposed methodology adopted

Antifungal susceptibility testing using NCCLS method M27A for *C. albicans*

The relative susceptibility of analogues against *Candida* isolate used in this study was determined using NCCLS 27A method [34-36] by modified microdilution test [37] in YEPD. Cells were grown for 48 h at 30 ^{0}C to obtain single colonies which were resuspended in a 0.9 % normal saline solution to give an optical density at 600nm (OD_{600}) of 0.1. The cells were then diluted 100 folds in YEPD media. The diluted cell suspensions were added to round bottomed 96-well microtiter plates (100 µl/ well) in wells containing equal volumes of medium (100 µl/ well) with different concentrations of drugs. Drug free control was also included. The plates were incubated at 30 ^{0}C for 48 h. The MIC test end point was evaluated both visually and by reading the OD_{620} in a microplate reader and is defined as the lowest drug concentration, which gave > 50 % inhibition of growth compared with drug free controls.

Antibacterial susceptibility testing

The relative susceptibility of analogues against *DH5α* and *ER2566* used in this study was determined using microdilution test in bacto agar. Cells were grown for 12 h at 37 ^{0}C to obtain single colonies which were resuspended in a 0.9 % normal saline solution to give an optical density at 600nm (OD_{600}) of 0.1. The cells were then diluted 100 folds in bacto agar media. The diluted cell suspensions were added to round bottomed 96-well microtiter plates (100 µl/ well) in wells containing equal volumes of medium (100 µl/ well) with different concentrations of drugs. Drug free control was also included. The plates were incubated at 37 ^{0}C for 48 h. The MIC test end point was evaluated both visually and by reading the OD_{620} in a microplate reader and is defined as the lowest drug concentration, which gave > 50 % inhibition of growth compared with drug free controls.

Comet Assay for DNA Double-strand Breaks Estimation

Lymphocytes were isolated from whole male wistar rat's blood using Histopaque 1077 as described by Pandey et al. [38]. Viability was determined by the Trypan blue dye-exclusion technique before conducting the Comet assay [39].Assay was performed according to the technique of Singh [40]. Immediately after the incubation period, a single-cell suspension was made by using pipette. From the suspension, 10 µl of suspension was mixed with 0.2 ml, 0.7% agarose. Agarose was suspended in phosphate buffered saline with 3:1 agarose higher resolution and kept at 37 °C to maintain physiological conditions [41]. The mixture was pipetted out and poured onto a fully frosted slide, immediately covered with coverglass (24×60 mm). These slides were kept in an ice-cold steel tray on ice for 1 min to allow the agarose to gel. Again, a layer was made over the gel with 100 µl of agarose as before, after removing the coverglass [40, 42]. These slides were immersed in ice-cold lysing solution and kept for 2 hours at 4 °C. After lysing, the slides were removed and placed in a horizontal slab of an electrophoresis assembly. One liter of electrophoresis buffer was gently poured into the assembly. After 20 min to allow for unwinding, electrophoresis was started at 250 mA (12 V) for 30 min.

The slides were removed from the electrophoresis apparatus and placed in coplin jar containing neutralizing buffer. After 30 min, the slides were transferred to another jar of neutralizing solution. After one more change of 30 min, the slides were left vertical at room temperature to dry and stained with ethidium bromide (EtBr of 0.05 mg/ml) covered with a 24×60-mm coverglass. Microscopic slides were prepared with each individual drug separately. Images were taken at 100× magnification using a charge-coupled device camera GW525x (Genwac, Orangeburg, NY, USA) attached to Leica DMLB fluorescence microscope (Leica, Wetzlar, Germany) with an excitation filter of 490 nm, a 500-nm dichroic filter, and an emission filter of 515 nm. The images of double strand DNA break in lymphocytes were recorded with fluorescence microscope.

In silico **analysis of the analogs**

Lipinski's Rule of Five and ADME prediction of the analogues

The structures of the two synthesized analogs were drawn in Chemdraw and the smile-id was generated. The generated smile-id was submitted in online available (http://www.organic-chemistry.org/prog/peo/) portal for analysing the potency of the lead molecule using Lipinski's Rule of Five [43]. The mol files of the analogues were submitted in the online available portal **http://preadmet.bmdrc.org/** to get an idea about the ADME (absorption distribution, metabolism and excretion) potential of the drugs.

Docking Studies

Docking experiments were performed using the AutoDock Tools 4.0 [44- 46] the most commonly cited docking program in the scientific literature [47] developed at the Scripps Research Institute, Molecular Graphics Laboratory, USA. The tool uses a genetic algorithm to find the preferred binding conformations of the ligand in the receptor. (http://autodock.scripps.edu). The Docking methodology involved the preparation of receptor and ligand molecules, docking using a Search algorithm and analysis of the binding conformation using a scoring function. Crystal structures of protein targets 14α sterol demethylase (bacterial target),reverse transcriptase(viral target),CaMdr1(fungal target), were retrieved from Brook Haven Protein Data Bank (www.pdb.org) and the heterologous atoms found in complex were removed to get the protein in its individual orientation.

RESULTS AND DISCUSSION

Anti-microbial potential of the analogues

The synthetic analogues were analysed for their potential to inhibit the growth of bacterial and fungal cultures in vitro. Both the analogues resulted in significant antibacterial activity against bacterial strain *ER2566* with MIC_{50} values of 31.25µg/ml for analogue 1 and 66.19µg/ml for analogue 2 as compared to standard curcumin (Figure 3).Both the analogues were again checked for their efficacy to act as potential anti-bacterial drug leads against bacterial strain *DH5α* and the results clearly replicate the one obtained against *ER2566* with both the analogue proving to be better that the standard used. Analogue 1 had MIC_{50} value of 21.72µg/ml while analogue 2 gave MIC_{50} 17.69µg/ml as compared to the standard curcumin (Figure 4).The significant and potent anti-bacterial activity of these can be explained attributing to the fact that extensive *in silico* studies performed at University of Lousiana,U.S.A to design molecules with increased activity and no cytotoxicity.Extensive research in the past several years have clearly showed that most of the drugs failed in the various stages of drug development inspite of having great potential to act as anti-microbial drugs because of the cytotoxic effects they showed on normal cells [32]. The analogs used in this study have been synthesized keeping in view the above mentioned fact and have been worked upon extensively using computational biology and bioinformatics tools to remove the functional groups responsible for cytotoxicity and retaining the activity of the parent compounds.

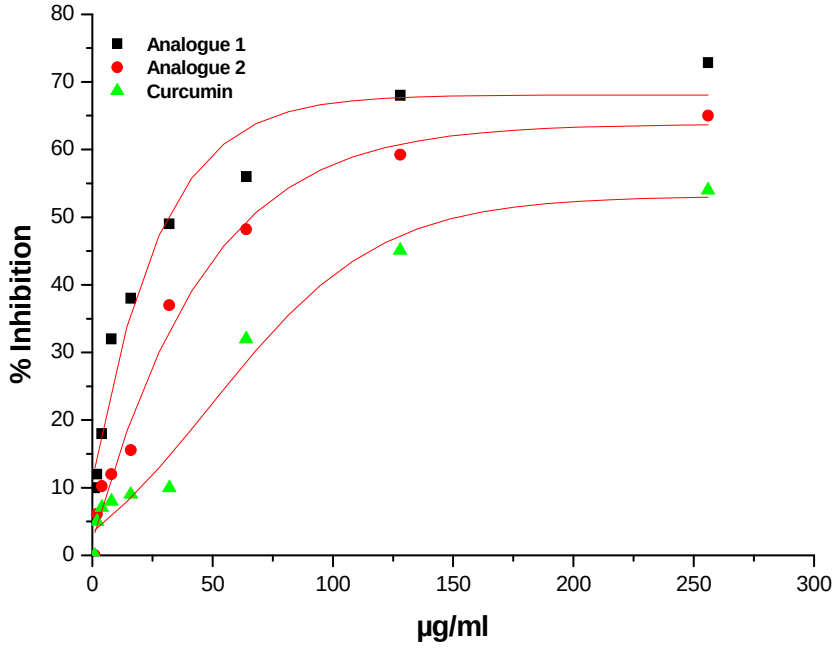

Figure 3: Antibacterial efficacy of analogues against ER2566 with both the analogues proving to be better in effect when compared to standard curcumin giving MIC_{50} values of 31.25µg/ml for analogue 1 (black) and 66.19µg/ml for analogue 2 (red) as compared to standard curcumin (green).

The analogues were further subjected to determine their antifungal potential against pathogenic fungus *Candida albicans*.The results clearly indicate that both the analogues are antifungal with MIC_{50} value of 170.50µg/ml for analogue 1 and 145.37µg/ml for analogue 2 but comparatively the results obtained from analogue 1 proved to be more significant as compared to analogue 2 and curcumin (Figure 5).

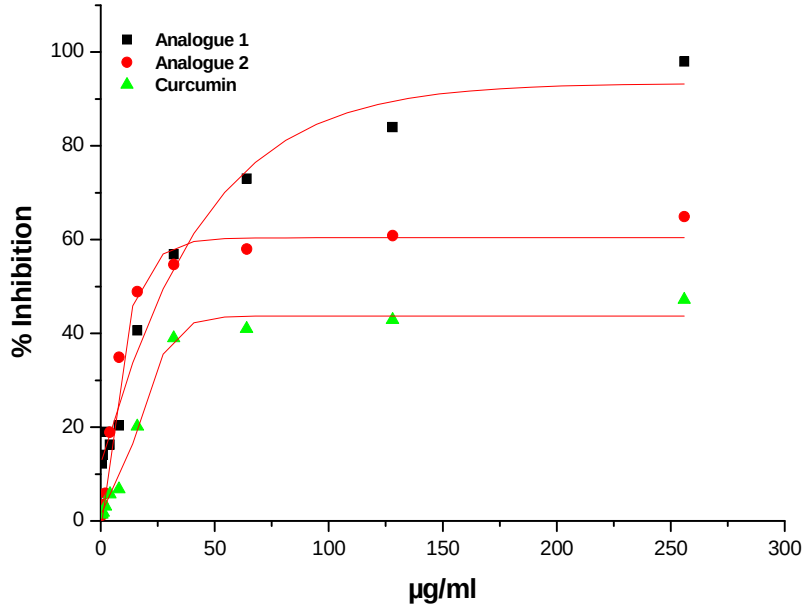

Figure 4: Antibacterial efficacy of analogues against DH5*α* with both the analogues proving to be better in effect when compared to standard curcumin giving MIC_{50} values of 21.72 µg/ml for analogue 1 (black) and 17.69 µg/ml for analogue 2 (red) as compared to standard curcumin (green).

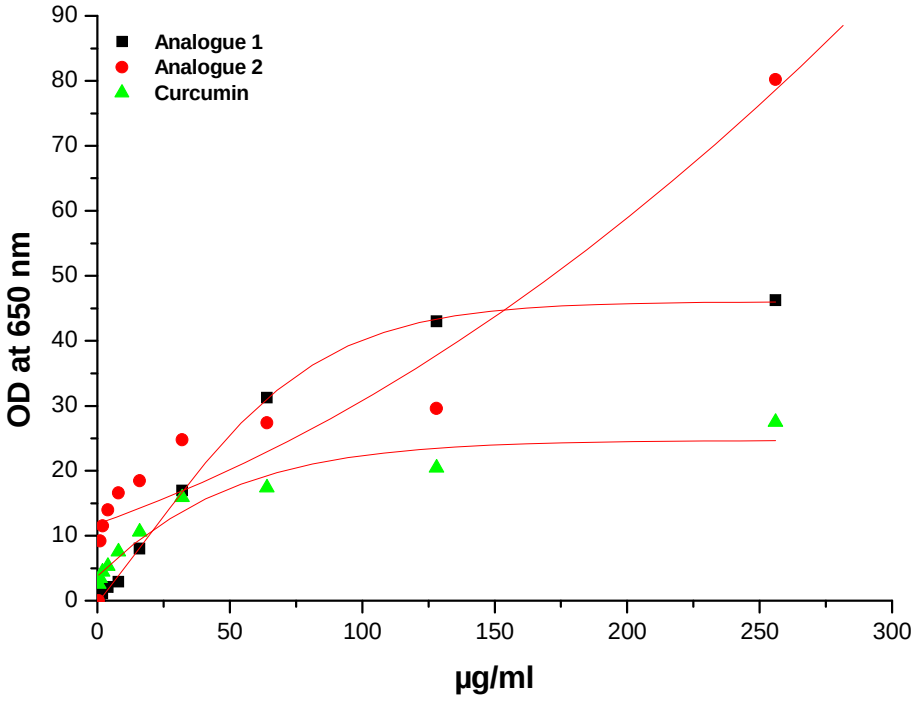

Figure 5: Antifungal efficacy of analogues against *Candida albicans* with both the analogues proving to be better in effect when compared to standard curcumin giving MIC_{50} value of 170.50 µg/ml for analogue 1 (black) and 145.37 µg/ml for analogue 2 (red) as compared to standard curcumin (green).

The anti-microbial efficacy of the synthesized analogues was reflected in fungus also but it is clearly evident from our results that MIC values are higher in fungus as obtained in the bacterial strains. This shift in the MIC values for fungus can be possibly explained by the existence of the

phenomena of multi drug resistance (MDR) in *Candida albicans* whereby the organism possesses various transporters or efflux pumps to efflux out the drug because of which may be more concentration of the analogues are required to kill the fungus when compared to bacteria.

Genotoxicity studies (Comet Assay) of the synthesized analogues

The inherent ability of the synthesized analogues having the potential to be genotoxic against extracted lymphocytes was analysed using the well known technique of comet assay. The results were found to be very promising with both the analogues were not genotoxic at a preliminary concentration of 308.95 µg/ml(100µM) for analogue 1 and 338.11µg/ml (100µM) for analogue 2 when incubated for 24 hrs(Figure 6).This was a very interesting finding because the idea behind synthesizing analogues from the parent compound was streamlined and strictly based in successful designing and subsequent synthesis of those molecules or leads which are different in structure to the parent compound but having the desirable property under investigation and most importantly having no cytotoxicity/genotoxicity against normal cells.

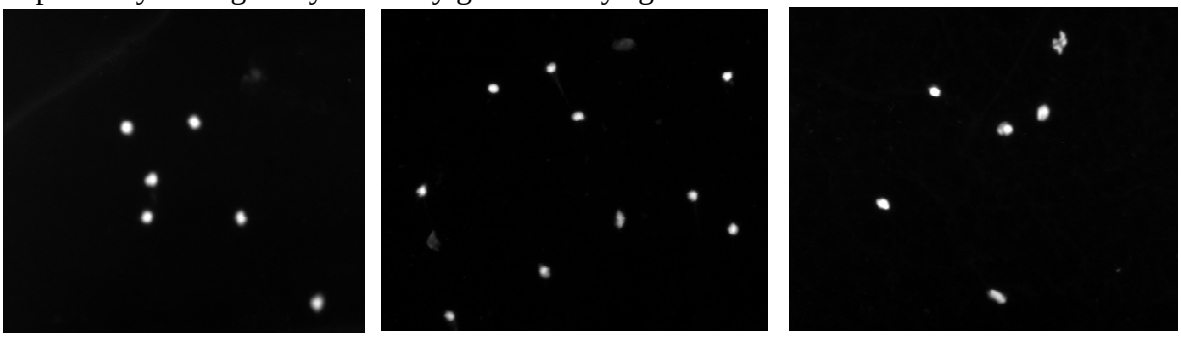

Figure 6: Genotoxic effect of the analogue 1 and 2 on extracted rat lymphocytes using comet assay. No double stranded DNA break is seen in all the treatments signifying the in-efficacy of the analogues to act as cytotoxic agents.

***In silico* studies of analogues**

Table 1: *In silico* studies of analogues by Lipinski's rule

Compound Name	Tumourogenic & Mutagenic Effect	Irritant	Reproductive effect	clogP	Solubility	Molecular weight	Drug Likeness	Drug score
Analogue 1	Both effects negative	Negative	Negative	1.86	-3.04	307	-1.9	0.5
Analogue 2	Both effects negative	Moderate	Negative	3.29	-3.39	336	-12.83	0.32

a) Analogues to have a reasonable probability of being well absorbed their logP value must not be greater than5.0.
b) More than 80% of the drugs on the market have a (estimated) logS value greater than - 4.
c) More than 80% of all traded drugs have a molecular weight below 450.
CLogP and LogS values obtained from http://www.organic-chemistry.Org/prog/peo/ portal.

In silico analysis of the two analogues using Lipinski's Rule of five gave rich results in terms of the ability of the synthesized analogues to qualify as efficient candidates for drug leads with both the analogues giving negative tumourogenic,mutagenic and reproductive effects as expected while analogue 1 had negative irritant effect as compared to moderate irritant effect of analogue 2.The probability of the analogues of being well absorbed their cLogP values must be less than 5.Both the analogues had clogP values in compliance with the Lipinsk's rule with analogue 1

having a value of 1.86 as compared to analogue 2 value of 3.86. The aqueous solubility of the compound which significantly affects its absorption and distribution characteristics is clearly evident from the fact that 80% of the traded drugs in the market have a solubility value greater than -4 and molecular weight less than 450.Both the analogues were having the potential of being well absorbed and distributed with solubility and molecular weight of -3.04,307 for analogue 1 and -3.39,336 for analogue 2(Table 1).The overall drug score for both the analogues was found to be positive stating that both the molecules contains predominantly fragments which are frequently present in commercial drugs. Therefore both the analogues were found to qualify the rule possessing desirable qualities present in the traded drugs.

Table 2: ADME (absorption, distribution, metabolism and excretion) prediction of analogues

Absorption			
S.No	**Name**	**Analogue 1**	**Analogue 2**
1	Human Intestinal Absorption (HIA, %)	93.52	95.92
2	In vitro Caco-2 cell permeability (nm/sec)	19.57	9.45
3	In vitro MDCK cell permeability (nm/sec)	0.45	0.34
4	In vitro skin permeability (log Kp.cm/hr)	-2.93	-1.50
Distribution			
1	In vitro plasma protein binding (%)	18.73	100.0
2	In vivo blood brain barrier penetration (C.Brain/C.Blood)	1.06	1.51

Statistical reports have shown that most of the drugs failed in the clinical trials because of the problems related to ADME because of which it is a major part of pharmacokinetics and is very essential for evaluation of compounds to qualify as drug candidates. (Beresford *et al.* 2002).Both the analogues resulted in giving good indications of being well absorbed with HIA % values of 93.52 for analogue 1 and 95.92 for analogue 2 in compliance with pre-requisite required 70 ~ 100 % for well absorbed compounds. The in-vitro Caco-2 cell permeability, MDCK cell permeability and skin permeability of the analogues were also evaluated and found to be satisfactorily good enough for both the drugs [48- 50]. The distribution pattern of the drugs was evaluated with the values of in-vitro plasma protein binding (%) because it is only the unbound drug which is available for diffusion or transport across cell membranes, and also for interaction with the pharmacological target and hence the plasma protein binding of the drug not only influences drug's action but also its deposition and efficacy. For potent compounds which are weakly bound and hence having more bioavailability, the analogue 1 was up to the task with %PPB values of 18.73 (< 90%) as compared to the analogue 2 which showed strong binding. The Blood Brain barrier penetration values which are of crucial importance in pharmaceutical sphere because in order to avoid CNS side effects the compounds must be CNS-inactive. For compounds having low absorption to CNS BB (C_{brain}/C_{blood}) values are less than 1.0 and compounds having middle absorption to CNS BB (C_{brain}/C_{blood}) values between 2.0 ~ 0.1(Table 2). Both the analogues were found to be moderate in terms of their efficacy to cross the blood brain barrier [51].

Docking studies of Analogues

To perform a preliminary evaluation of the binding efficiency of the analogues against selected anti-microbial targets and to propose a possible methodology before performing expensive and time consuming wet lab studies, the two analogues were docked against 14α sterol demethylase (bacterial target),reverse transcriptase(viral target),CaMdr1(fungal target).The binding efficacies of the analogues is evaluated in terms of binding energy which dictates the richness of binding and hydrogen bonds which are indicative of stability of binding. Both the analogues were giving good interactions with all the targets.

Analogues 1 & 2 giving binding energies of -4.16 and -5.11 with antibacterial target 14α sterol demethylase resulting in the formation of 1 H bond between hydrogen 18 of analogue 1 and threonine 264 of the target and 2 H bonds between H25 and O10 of analogue 2 with proline 386 and arginine 326 of target (table 3 and figure 7).

Table 3: Docking parameters of both the analogues when docked with bacterial protein 14α sterol demethylase

Compound Name	Binding Energy	Ligand Efficiency	Inhibition constant	Intermolecular energy	Electrostatic energy	Total internal energy	Hydrogen bonds
Analogue 1	- 4.16	- 0.32	898.06µM	- 5.01	- 0.06	- 0.27	1 H bond H18 – THR264
Analogue 2	- 5.11	- 0.34	179.29µM	- 6.35	- 0.23	- 0.4	2 H bonds H25-PRO386 & O10-ARG326

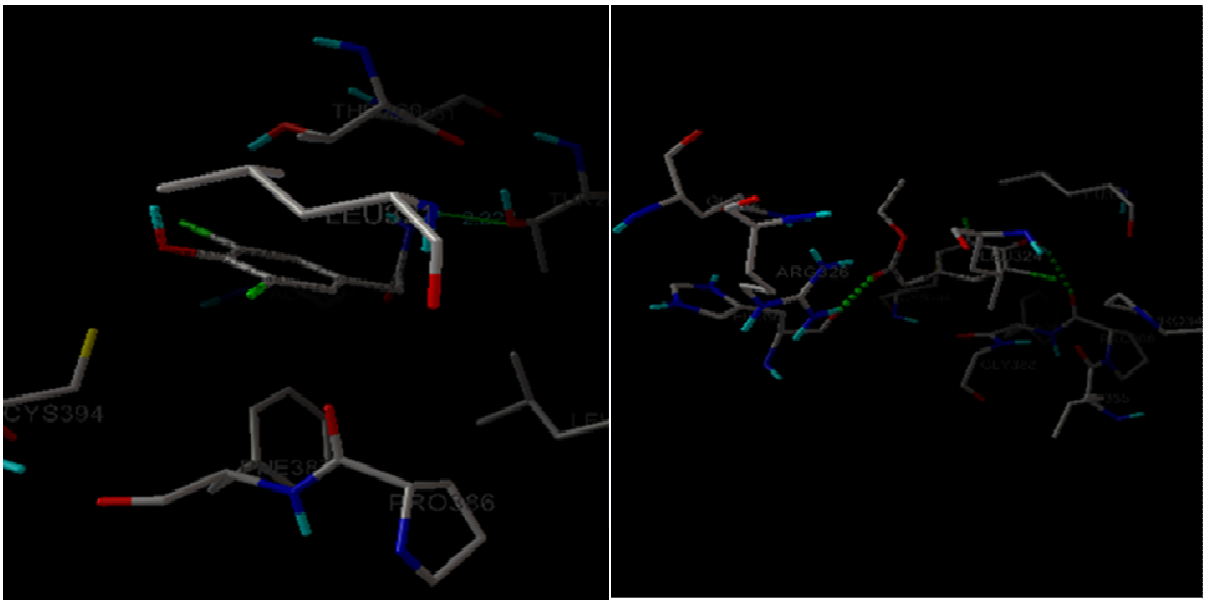

Figure 7: Analogue 1 and 2 docked with bacterial protein 14α sterol demethylase (dotted green lines show hydrogen bonds)

The binding energies of the two analogues obtained with viral target reverse transcriptase were -4.07(analogue 1) and -4.26 (analogue 2) respectively resulting in the formation of 2 H bonds between hydrogen17 and O8 of analogue 1 with asparginine 30 of the target and 2 H bonds between H25 and O10 of analogue 2 with glysine 48 and isoleucine 50 of target (table 4 and figure 8).

Table 4: Docking parameters of both the analogues when docked with viral protein reverse transcriptase

Compound Name	Binding Energy	Ligand Efficiency	Inhibition constant	Intermolecular energy	Electrostatic energy	Total internal energy	Hydrogen bonds
Analogue 1	- 4.07	- 0.31	1.03mM	- 4.83	- 0.18	- 0.37	2 H bonds H17 – ASP30 & O8-ASP30
Analogue 2	- 4.26	- 0.28	766.66µM	- 5.54	- 0.34	- 0.35	2 H bonds H25-GLY48 & O10-ILE50

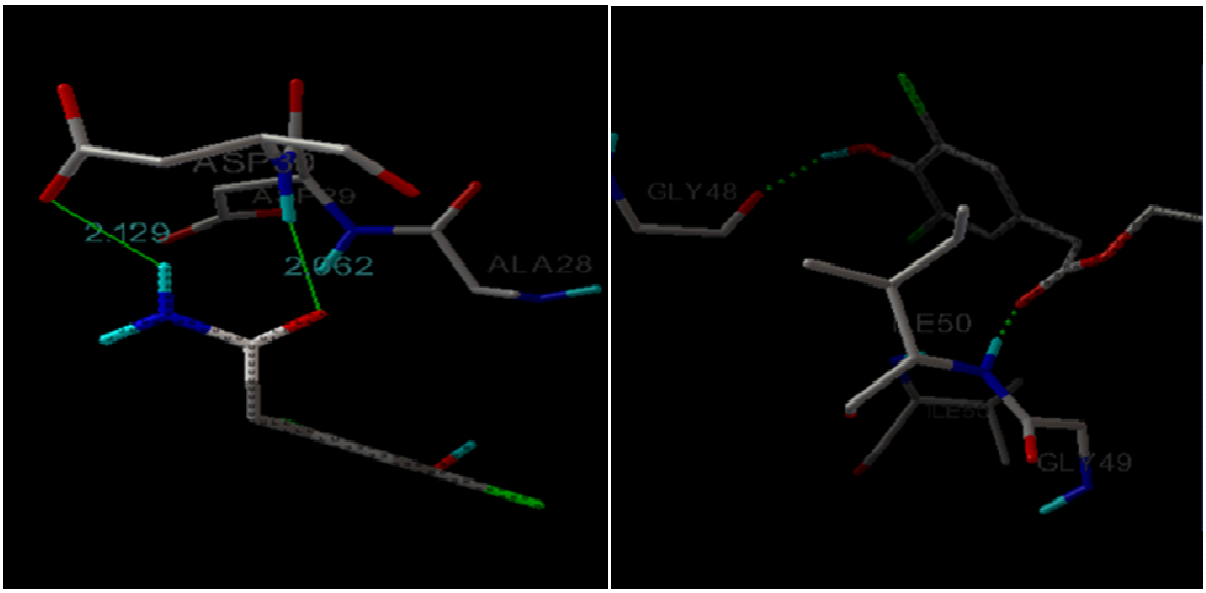

Figure 8: Analogue 1 and 2 docked with viral protein reverse transcriptase (dotted green lines show hydrogen bonds)

The binding energies of the two analogues obtained with fungal target efflux transporter were -5.13(analogue 1) and -5.39 (analogue 2) respectively resulting in the formation of 1 H bond between hydrogen17 of analogue 1 with glutamine 283 of the target and 1 H bond between O10 of analogue 2 with tyrosine 279 of target (table 5 and figure 9).

Table 5: Docking parameters of both the analogues when docked with anti-fungal protein CaMdr1

Compound Name	Binding Energy	Ligand Efficiency	Inhibition constant	Intermolecular energy	Electrostatic energy	Total internal energy	Hydrogen bonds
Analogue 1	- 5.13	- 0.39	173.92µM	- 5.94	- 0.19	- 0.31	1 H bond H17 – GLU283
Analogue 2	- 5.39	- 0.36	112.26µM	- 6.79	- 0.09	- 0.24	1 H bonds O10- TRY279

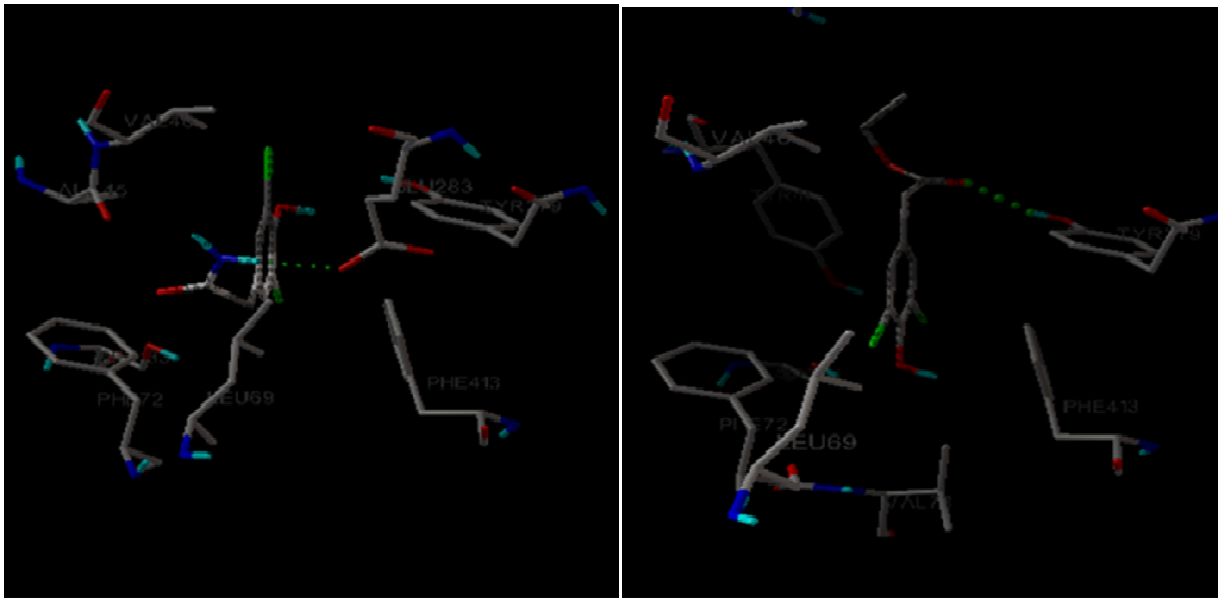

Figure 9: Analogue 1 and 2 docked with anti-fungal protein CaMdr1 (dotted green lines show hydrogen bonds)

CONCLUSION

Our preliminary results seem to indicate that the analogue 2 [ethyl 2-(3, 5-dibromo-4-hydroxyphenyl) acetate] exhibited better antimicrobial potentials compared to the analogue 1. These analogues also showed moderate drug likeliness as observed by Lipinski's rule of five and ADME. Further, no genotoxicity in the COMET assay prompted us to synthesize various analogues of these compounds which are being tested using various parameters**.**

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REFERENCES

[1] McConnell, O.J., Longley, R.E., and Koehn, F.E **(1994)**, In: Gullo, V.P., (Ed.), *The Discovery of Natural Products with Therapeutic Potential*. Butterworth-Heinemann, Boston, pp. 109-174.
[2] Donaldson, J., Kruger, E., Lindroth, R **(2006)**, *New Phytologist*, 169: 561-570.
[3] Blunt, J., Copp, B., Hu, W., Munro, M., Northcote, P., Prinsep, M **(2008)** *Nat Prod Rep*, 25: 35-94
[4] Paul, V., Puglisi, M **(2004)**, *Nat. Prod. Rep.* 21:189-209.
[5] Paul, V., Puglisi, M., Ritson-Williams, R **(2006),** *Natural Product Reports*, 23:153-180.
[6] Cimino, G., De Rosa, S., De Stefano, S., Self, R., Sodano, G **(1983)**, *Tetrahedron Letters*, 24: 3029-3032.
[7] Ciminiello, P., Costantino, V., Fattorusso, E., Magno, S., Mangoni, A **(1994a)**, *J Nat Prod*. 57:705-712
[8] Ciminiello, P., Fattorusso, E., Magno, S **(1994b)**, *J Nat Prod*, 57:1564-1569.
[9] Ciminiello, P., Fattorusso, E., Magno, S **(1995),** *J Nat Prod* 58:689-696.
[10] Ciminiello, P., Dell,`Aversano, C., Fattorusso, E., Magno, S **(1996a)**, *Tetrahedron*, 52:9863-9868
[11] Ciminiello, P., Fattorusso, E., Magno, S., Pansini, M **(1996b)**, *Biochemical Systematics and Ecology*, 24: 105-113.
[12] Ciminiello, P., Fattorusso, E., Forino, M., Magno, S **(1997)**, Tetrahedron, 53: 6565-6572
[13] Ciminiello, P., Dell,`Aversano, C., Fattorusso, E., Magno, S., Pansini, M **(1999)**, *J Nat Prod*, 62:590-593
[14] Ciminiello, P., Dell Aversano, C., Fattorusso, E., Magno, S., Pansini, M **(2000)**, *J Nat Prod* 63:263-266
[15] Thoms, C., Wolff, M., Padmakumar, K., Ebel, R., Proksch, P **(2004)**, *Z Naturforsch [C]*, 59:113-122.
[16] Teeyapant, R., Woerdenbag, H.J., Kreis, P., Hacker, J., Wray, V., Proksch, P **(1993b)**, *Z Naturforsch [C]*, 48: 939-945
[17] Weiss, B., Ebel, R., Elbrächter, M., Kirchner, M., Proksch, P **(1996)**, *Biochemical Systematics and Ecology*, 24:1-12.
[18] Encarnación-Dimayuga, E., Ramírez, M., Luna-Herrera, J **(2003)**, *Pharmaceutical Biology*, 41:384-387.
[19] Pawlik, J., Chanas, B., Toonen, R., Fenical, W **(1995)**, *Mar Ecol Prog Ser*, 127:183- 194.
[20] Pansini, M. **(1997)**, *Biol Mar Mediterr*, 4:74-80.
[21] Thoms, C., Ebel, R., Hentschel, U., Proksch, P **(2003a),** *Z Naturforsch [C]*, 58:426- 432.
[22] Thoms, C., Horn, M., Wagner, M., Hentschel, U., Proksch, P **(2003b)**, *Marine Biology*, 142:685-692.
[23] Wilkinson, C., Vacelet, J **(1979),** *Journal of Experimental Marine Biology and Ecology*, 37:91-104.
[24] Borkholder, P. R. & Ruetzler,K. (**1969)** *Nature, New Biol.* 222,983-984.
[25] Quindós, G. **(2002)** *Rev. Iberoam. Micol.* 19: 1-4.
[26] García-Ruiz, J.C., Amutio, E. and Pontón, J **(2004)** *Rev. Iberoam. Micol.* 21: 55-62.
[27] Pontón, J., Rüchel, R., Clemonds, K.V., Coleman, D.C., Grillot, R., Guarro, J., Aldebert, D., Ambroise-Thomas, P., Cano, J., Carrillo- Muñoz, A.J., Gené, J., Pinel, C., Stevens, D.A. and Sullivan, D.J. **(2000)** *Med. Mycol.* 38(S1): 225-236.
[28] Sandven, P. **(2000)** *Rev. Iberoam. Micol.* 17: 73-81.
[29] Walsh, T.J., Groll, A., Hiemenz, J., Fleming, R., Roilides, E. and Anaissie, E. **(2004),** *Clin. Microbiol. Infect.* 10(S1): 48-66.
[30] Giusiano, G., Mangiaterra, M., Rojas, F. and Gámez, V. **(2005)**, *J. Chemother.* 17: 347-350.

[31] Giusiano, G., Mangiaterra, M., Rojas, F. and Gámez, V. (2004) *Mycoses*; 47: 300-303.
[32] Donia, M. and Hamann, M. **(2003)**, *Lancet Infect. Dis.* 3: 338-48.
[33] Sallam**,** A.A., Ramasahayam**,** S., Meyer**,** S.A. and El Sayed, K.A **(2010)**, *Bioorganic & Medicinal Chemistry* (18)21:7446-7457.
[34] Espinel-Ingroff, A., Barchiesi, F., Hazen, K.C., Martinez-Suarez, J.V. & Scalise, G **(1998)**, *Medical Mycology, Supplement*, vol. 36, no. 1, pp. 68-78.
[35] Martins, M.D., Rex, J.H **(1997)**, *Clinical Updates in Fungal Infections* vol. 1, pp. 1-6.
[36] Rex, J.H., Pfaller, M.A., Galgiani, J.N., Bartlett, M.S., Espinel-Ingroff, A., Ghannoum, M.A., Lancaster, M., Odds, F.C., Rinaldi, M.G., Walsh, T.J. & Barry, A.L **(1997)**, *Clinical Infectious Diseases*, vol. 24, no. 2, pp. 235-249.
[37] Talibi, D. & Raymond, M **(1999)**, *Journal of Bacteriology*, vol. 181, no. 1, pp. 231-240.
[38] Pandey, A.K., Bajpayee, M., Parmar, D., Rastogi, S.K., Mathur, N., Seth, P.K., Dhawan, A **(2005),** *Environ Mol Mutagen*, 45:435–441.
[39] Phillips, H.J. **(1973)**, *Dye exclusion tests for cell viability. In: Kruse PF, Patterson MJ, editors. Tissue Culture: Methods and Applications. New York: Academic Press*, 406- 408.
[40] Singh, N. **(2003)**, In R. Blumenthal (Ed.), Apoptosis by DNA diffusion assay, methods in molecular medicine (chemosensitivity). Totowas: Humana.
[41] Lai, H., & Singh, N.P. **(1996)**, *International Journal of Radiation Biology*, 69:513–521.
[42] Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H **(2000),** *Environmental and Molecular Mutagenesis*, 35:206–221.
[43] Khan, M.S., Siddiqui, S.A., Siddiqui, M.S., Goswami, U., Srinivasan, K.V. and Khan, M.I **(2008)**, *Chem Biol Drug Des*. 72(3):197-204.
[44] Morris, G.M., Goodsell, D.S., Huey, R. and Olson, A.J. **(1996)**, *J Comput Aided Mol Des.* 10: 293-304.
[45] Morris G.M., Goodsell, D.S., Halliday, R.S., Huey, R., Hart, W.E., Belew, R.K. and Olson, A.J. **(1998),** *J Comput Chem.* 19: 1639-1662.
[46] Goodsell, D.S., Olson, A.J. **(1990)**, *Proteins*, 8: 195-202.
[47] Sousa, S.F., Fernandes, P.A., Ramos, M.J. **(2006)**, *Proteins*, 65: 15-26.
[48] Yamashita,S.,Furubayashi,T.,Kataoka,M.,Sakane,T.,Sezaki,H.and Tokuda,2000*Eur. J. Pharm. Sci. 10*:195.
[49] Irvine, J.D., Takahashi, L., Lockhart, K., Cheong, J., Tolan, J.W., Selick, H.E., Russelo, G., **1999**. *J. Pharm. Sci*. 88: 28–33.
[50] Singh,S.,Singh,J., *Med. Res. Rev*. **1993**, *13*, 569.
[51] Ma X.,Chen,C.,Yang,J., *Acta Pharmacologica Sinica.* **2005**, *26*, 500.