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СИНТЕЗ ТРИАЗОЛСОДЕРЖАЩЕГО КОНЪЮГАТА ЦИПРОФЛОКСАЦИНА И *in silico* ТЕСТИРОВАНИЕ ЕГО КАК ЛИГАНДА ЦИТОХРОМОВ Р450

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Цитохромы Р450 – это гемсодержащие монооксигеназы, которые катализируют реакции биосинтеза различных соединений, важных для нормального функционирования клеток, превращения лекарств и ксенобиотиков. Одни цитохромы Р450 (например, CYP19 и CYP17 человека, CYP51 грибков) являются белками-мишенями ряда лекарственных соединений, другие представляют интерес для фармакологических исследований. Для создания нового флуоресцирующего ингибитора цитохромов Р450 был получен азолсодержащий конъюгат ципрофлоксацина (CPF-bab-Z1). В целях оценки потенциала этого соединения в качестве лиганда для цитохромов Р450 проведен высокопроизводительный виртуальный скрининг CPF-bab-Z1 и множества известных 3D-структур Р450. Среди 28 структур цитохромов CYP51 наилучшая аффинность обнаружена у белка 5esh (минимальное значение энергии связывания составило –12,5 ккал/моль). Положение CPF-bab-Z1, рассчитанное в активном центре этого белка, характеризуется близостью циклопропильного (не азольного) фрагмента к гемовому железу CYP51. Полученные данные показывают перспективы исследований CPF-bab-Z1 *in vitro* с цитохромами Р450.

Ключевые слова: Р450; флуоресценция; азолы; ингибиторы; докинг; ципрофлоксацин.

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SYNTHESIS OF TRIAZOLE-CONTAINING CIPROFLOXACIN CONJUGATE AND ITS *in silico* TEST AS A CYTOCHROME P450 LIGAND

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Cytochromes P450 are hem-containing monooxygenases which catalyse biosynthesis of many compounds playing an essential role in cellular functions as well as degradation of drugs and xenobiotics. Some P450s (e. g., human CYP19 and CYP17, fungal CYP51) are valid target proteins for some drugs. The others P450s are also interesting for pharmacology-related researches. Aiming to design new fluorescent inhibitor of P450s we have synthesised the azole-bearing conjugate of ciprofloxacin (CPF-bab-Z1). To estimate potential of the compound as a ligand for CYPs we performed high-throughput virtual screening (multiple docking calculations) for CPF-bab-Z1 and multiple known 3D structures of P450s. The best affinity for CPF-bab-Z1 (the smallest value of energy of binding is equal –12.5 kcal/mol) were found for protein with PDB code 5esh among 28 structures of CYP51. The calculated pose of CPF-bab-Z1 in the active site of the protein is characterised by cyclopropyl (but not azole) proximity to the heme iron of the CYP51. The data obtained demonstrate perspectives for *in vitro* investigations of CPF-bab-Z1 with P450s.

Keywords: P450; fluorescence; azoles; inhibitors; docking; ciprofloxacin.

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Introduction

Cytochromes P450 (or CYPs) are a family of heme-containing monooxygenases. CYPs catalyse versatile set of oxidative reactions resulting in biosynthesis of essential metabolites, bioconversion of drugs and other xenobiotics [1]. Some CYPs are known to be valid targets for specific drugs (e. g., human CYP17 and CYP19 [2], fungal CYP51 [3]) and a lot of others are also considered to be promising in this respect or as drugs' degrader [1]. Many synthetic medicinal inhibitors of CYPs contain an azole moiety, which is essential for formation of strong coordination N-Fe bond with iron ion from the enzymes' heme moieties. Formation of the N-Fe bond impacts on inhibition efficiency *via* enhancing of such inhibitors binding (and, thus, preventing specific substrates binding) and disabling of the iron ion binding with dioxygen (the common co-substrate for CYPs) [4]. New azole-bearing compounds with the property of a CYP inhibitor are of interest due to growth of resistance to existing azole-containing drugs [5]. On the other hand, fluorescent compounds are convenient for detection in complex biological matrices. Many of them are molecular probes to study a metabolism, distribution and protein interactions in various biological samples (proteins, cells and even multicellular organisms). To the best of our knowledge, a few fluorescent azole-bearing antifungal drug analogues were reported which both retain the anti-fungal activity of their prototypes and can stain cell structures, likely due to their hydrophobic cation moieties with a tropism to the organelles (Cy to mitochondria, diethylamino-coumarin-carboxamide to endoplasmic reticulum) [6; 7]. Thus, aiming to design new fluorescent inhibitor of CYPs we have synthesized the azole-bearing conjugate of ciprofloxacin ((7-(4-(2-(4-((1H-1,2,4-triazol-1-yl)methyl) phenylamino)-2-oxoethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid), CPF-bab-Z1) due to a known ability of the anti-bacterial drug to fluoresce with blue light (excitation and emission maxima at 280 and 450 nm respectively) [8]. To estimate potential of the compound as a ligand for CYPs we have performed high-throughput virtual screening (multiple docking calculations) for the structure and multiple known 3D structures of CYPs from various organisms.

Experimental section

Ciprofloxacin hydrochloride (CPF · HCl), bromoacetyl bromide, pyridine (Py), 4-(1H-1,2,4-triazol-1-ylmethyl)aniline hydrochloride (Z1 · HCl), silica gel for chromatography (*Sigma-Aldrich*, USA), $NaHCO_3$ (*Bashkir soda company*, Russia), acetonitrile (AcN), methanol (MeOH) (*Merck*, USA) were used. Synthesis of CPF-bab-Z1 was performed according to the scheme depicted below (fig. 1).

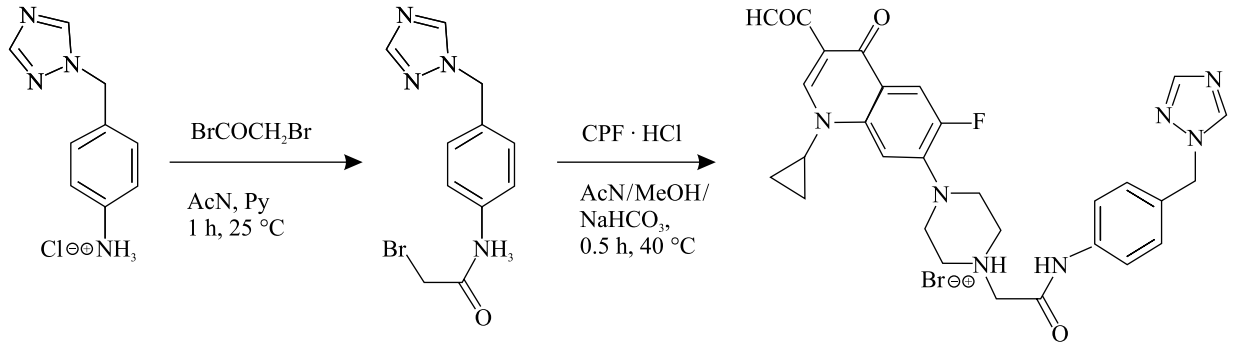

Fig. 1. A scheme of synthesis of CPF-bab-Z1

Solution of bromoacetyl bromide (1.05 eq) in acetonitrile was added dropwise to a stirred solution of Z1 · HCl (1 eq) in acetonitrile with Py (3 eq). The mixture was stirred for 1 h at room temperature (~20 °C) and then filtered using cotton wool and rotary evaporated giving yellowish solid. Then the residue were dissolved in MeOH : AcN (1 : 1, v : v) and mixed with $NaHCO_3$ (5 eq) and a suspension of CPF · HCl (1 eq) in MeOH. The mixture was stirred for 30 min at 40 ºC until forming clear solution. Then the solution was filtered using cotton wool and evaporated giving off-white solid. The solid was dissolved in MeOH and purified by column chromatography using AcN : MeOH (1 : 1, v : v).

High performance liquid chromatography (HPLC) analysis was performed using Agilent liquid chromatograph, column Poroshell 120 EC-C18 (75 × 4.6 mm, 2.7 μm), elution at 30 ºC with flow rate 0.5 mL/min using a gradient of H_2O : MeOH in a range of 5–100 % MeOH. Mass-spectrometric analysis was carried out using LC-MS-2020 system as described [9] with AcN as eluent and MeOH for sample dilution.

AutoDock Vina [10] software was used for virtual screening; grid centers for all calculations were 4 × 4 × 4 nm with their centers at geometrical centers of correspondent CYPs, exhaustiveness was set to 12. In general, we have processed 28 structures of CYP51 from various organisms, 73 structures of other mycobacterial CYPs and 185 structures of human CYPs. Values of energy of binding (E_{bind}), the amino acids surrounding a ligand and poses with close triazole – heme iron have been highlighted, tabulated and discussed.

Results and discussion

The compound CPF-bab-Z1 is a triazolomethylaniline conjugated with ciprofloxacin via their amino-groups using —CO—CH_2— linker derived from bromoacetyl bromide (see fig. 1). ESI-MS spectrum of the compound was obtained (fig. 2), confirming the desired molecular weight (m/z of the mass-to-charge ratio $[M + H]^+$ for $C_{28}H_{29}FN_7O_4^+$ is equal 546.23; found 546.20); CPF-bab-Z1 purity was found to be ~95 % by HPLC data (fig. 3).

CPF-bab-Z1 is also a azole and, thus, it is interesting as a potential fluorescent inhibitor of CYPs. Thus, to rationalise further experimental investigations we performed *in silico* screening for a large set of structures of CYPs using computational docking. First, we tested CYP51 structures due to it is a target for antifungal azoles and the most studied CYP [3]. Docking results for CYP51 set are summarised in tables 1 and 2.

Table 1

E_{bind} values for CPF-bab-Z1 *in silico* interactions with structures CYP51 of non-pathogenic baker yeast *Saccharomyces cerevisiae*

PDB code	Data	PDB code	Data	PDB code	Data
4lxj	–11.9	5ead	–11.8; Fe	5esi	–9.3
4wmz	–11.3	5eae	–11.9; Fe	5esj	–11.5
4zdy	–11.8; Fe	5eaf	–9.3	5esk	–12.3; Fe
4zdz	–10.8	5eag	–10.7; Fe	5esl	–11.8; Fe
4ze0	–11.6; Fe	5eah	–9.3; Fe	5esm	–11.9; Fe
4ze1	–12.1; Fe	5eqb	–11.7; Fe	5esn	–11.2
4ze2	–11.1	5ese	–12.4; Fe	5hs1	–8.6
4ze3	–11.9; Fe	5esf	–12; Fe	5ul0	–11.4; Fe
5eab	–11.3; Fe	5esg	–11.9; Fe	6e8q	–11.9; Fe
5eac	–11.8; Fe	**5esh**	**–12.5; Fe**	–	–

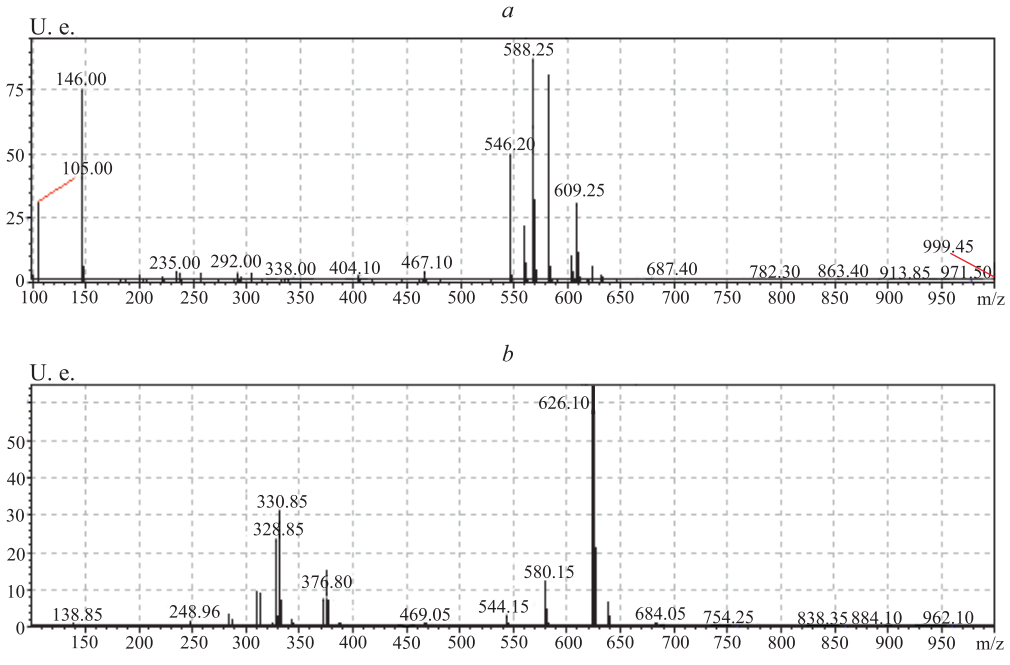

Fig. 2. ESI-MS spectra of CPF-bab-Z1 in positive (*a*) and negative (*b*) ions registration modes. Interpretation of signals (cations: $[M + H]^+$ 546.20, $[M + Na]^+$ 568.21, $[M + K]^+$ 584.20, $[M + ACN + Na]^+$ 609.24; anions: $[M + Br]^-$ 626.10 and 624.00, $[M + Cl]^-$ 580.20, $[M – H]^-$ 544.20)

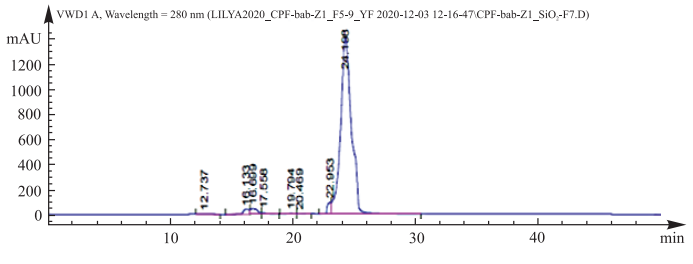

Fig. 3. HPLC chromatogram of CPF-bab-Z1 (RT = 24.2 min)

CPF-bab-Z1 demonstrates the minimal value of E_{bind} (–12.5 kcal/mol) for *Saccharomyces cerevisiae* CYP51 structure with PDB code 5esh, but the overage value of E_{bind} has been –11.3 kcal/mol for all structures considered for the same enzyme. In the predicted complex with CYP51 (5esh) not azole, but cyclopropyl ring of CPF-bab-Z1 is close to Fe of the structure heme. This indicates the compound behaves *in silico* as a substrate, but not like azole inhibitor, of the enzyme. Cyclopropyl ring oxidation could cause formation of reactive intermediate, which could attach covalently to an amino acid residue of such enzyme or to glutathione resulting in its depletion *in cellulo* [11]. Poses with triazole proximity to heme have also been found for some *Saccharomyces cerevisiae* CYP51 structures (4zdz, 4ze2, 5esjcA, etc.), but in every case the N-Fe bond formation has been prohibited due to distance between the atoms. Results of the docking for CYP51 from pathogenic microbes are in table 2.

Table 2

E_{bind} values for CPF-bab-Z1 *in silico* interactions with structures CYP51 of pathogenic microorganisms

CYP of organism	PDB code	Data	CYP of organism	PDB code	Data
CYP51 *Aspergillus fumigatus*	6cr2	–11.6	CYP51 *Mycobacterium tuberculosis*	1e9x	–10.7
CYP51 *Aspergillus fumigatus*	5frb	–11.6; Fe	CYP51 *Mycobacterium tuberculosis*	2vku	–9.9

Ending table 2

CYP of organism	PDB code	Data	CYP of organism	PDB code	Data
CYP51 *Aspergillus fumigatus*	4uym	–11.4; Fe	CYP51 *Mycobacterium tuberculosis*	1h5z	–9.8; Fe
CYP51 *Aspergillus fumigatus*	4uyl	–10.6; Fe	CYP51 *Mycobacterium tuberculosis*	1u13	–9.6
CYP51 *Candida albicans*	5fsa	–12; Fe	CYP51 *Mycobacterium tuberculosis*	1x8v	–9.5
CYP51 *Candida albicans*	5v5z	–11.3; Fe	CYP51 *Mycobacterium tuberculosis*	2bz9	–9.4; Fe
CYP51 *Candida albicans*	5tz1	–11.2; Fe	CYP51 *Naegleria fowleri*	5tl8	–11.2; Fe
CYP51 *Candida glabrata*	5jlc	–11	CYP51 *Naegleria fowleri*	6ayc	–11.1
CYP51 *Leishmania infantum*	3l4d	–10.7; Fe	CYP51 *Naegleria fowleri*	6ay6	–10.9
CYP51 *Mycobacterium tuberculosis*	2w0b	–12	CYP51 *Naegleria fowleri*	6ayb	–10.8
CYP51 *Trypanosoma brucei*	4g3j	–10.5	CYP51 *Trypanosoma cruzi*	3khm	–10.7; Fe
CYP51 *Trypanosoma brucei*	3p99	–10.3	CYP51 *Trypanosoma cruzi*	4ck9	–10.7
CYP51 *Trypanosoma brucei*	2x2n	–10.3	CYP51 *Trypanosoma cruzi*	3k1o	–10.5
CYP51 *Trypanosoma brucei*	3g1q	–9.2	CYP51 *Trypanosoma cruzi*	3zg3	–10.4; Fe
CYP51 *Trypanosoma brucei*	3tik	–9.7; Fe	CYP51 *Trypanosoma cruzi*	4ck8	–10.3; Fe
CYP51 *Trypanosoma brucei*	4g7g	–9; Fe	CYP51 *Trypanosoma cruzi*	4c27	–10.3
CYP51 *Trypanosoma cruzi*	6fmo	–11.8	CYP51 *Trypanosoma cruzi*	4bmm	–10.2; Fe
CYP51 *Trypanosoma cruzi*	2wuz	–11.4; Fe	CYP51 *Trypanosoma cruzi*	4uqh	–10.2; Fe
CYP51 *Trypanosoma cruzi*	2wx2	–10.9; Fe	CYP51 *Trypanosoma cruzi*	4coh	–9.8; Fe
CYP51 *Trypanosoma cruzi*	4h6o	–10.9	CYP51 *Trypanosoma cruzi*	4by0	–9.8
CYP51 *Trypanosoma cruzi*	4cka	–10.7; Fe	CYP51 *Trypanosoma cruzi*	3ksw	–9.6; Fe

For the predicted docking positions with the lowest E_{bind} values indicated in table 2 in the frames of one microorganism a cyclopropane-to-heme orientation has been found for CYP51 from *Aspergillus fumigatus* (5frb), *Candida albicans* (5fsa), formally for *Mycobacterium tuberculosis* (2w0b) and «brain-eating» amoeba *Naegleria fowleri* (5tl8). Classical azole-to-heme oriented positions have been found for CYP51 from *Leishmania infantum* (3l4d), *Trypanosoma brucei* (4g3j) and *Trypanosoma cruzi* (2wuz) (fig. 4).

CPF-bab-Z1 has demonstrated E_{bind} within the range of –11.1 kcal/mol (CYP121, 4ktj) to –7.5 kcal/mol (CYP119, 4yof); mean E_{bind} for all mycobacterial CYPs tested was found to be ~9.5 kcal/mol, excepting CYP51. Poses with azole-to-heme orientation were rare, e. g., for PDB code 2x5w, 5li7, 2wh8 and 6dcd are among good ones; the top 12 lowest E_{bind} for the ligand and the enzymes' structures are in the table 3.

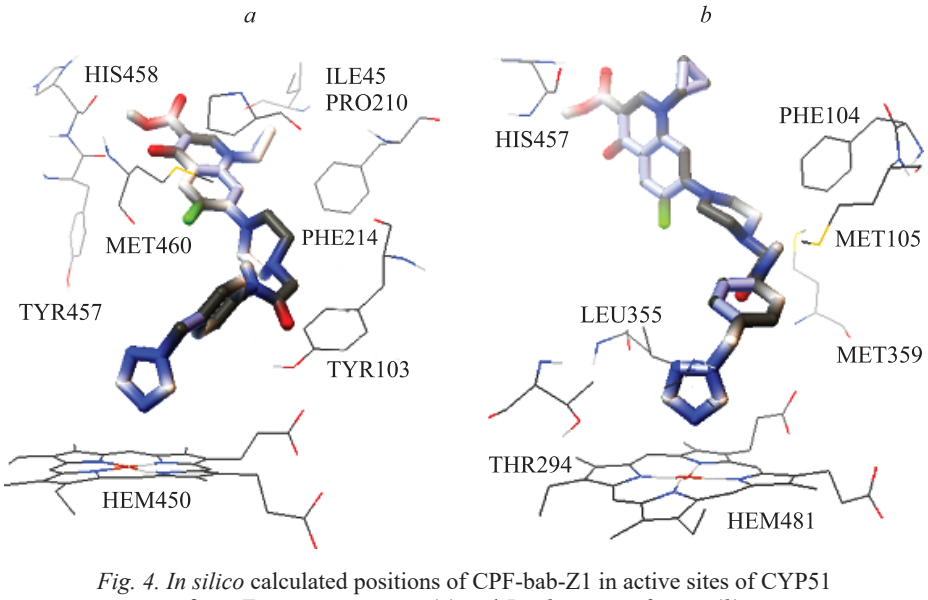

Fig. 4. In silico calculated positions of CPF-bab-Z1 in active sites of CYP51 from *Trypanosoma cruzi* (*a*) and *Leishmania infantum* (*b*)

Table 3

Top 12 E_{bind} values for CPF-bab-Z1 *in silico* interactions with structures of CYPs from mycobacteria, excepting CYP51

PDB code	CYP	E_{bind}, kcal/mol	Fe	N41
4ktj	CYP121	–11.1	–	N41-ALA167, ALA178, ASN181, TRP182
5o4k	CYP121	–11.1	–	N41-ALA167, ALA178, ASN181, TRP182
2wm4	CYP124	–11.0	Fe	N41-THR271, VAL315
2x5w	CYP125	–10.9	Fe	N41-ALA268, HEM431
3r9b	CYP164A2	–10.9	Fe	N41-ALA256
4g48	CYP121	–10.9	–	N41-ALA167, ALA178, ASN181, TRP182
4ict	CYP121	–10.9	–	N41-ALA167, ALA178, ASN181, TRP182
4ktf	CYP121	–10.9	–	N41-LEU160, ASP185, PHE231
5opa	CYP121	–10.9	–	N41-LEU160, LEU164, ASP185, PHE231
3g5h	CYP121	–10.8	–	N41-ALA167, ALA178, ASN181, TRP182
5li7	CYP126A1	–8.5	Fe	N41-THR257, HEM501
2wh8	CYP130	–9.2	Fe	N41-GLY243, HEM450

CPF-bab-Z1 has demonstrated E_{bind} from –12.3 kcal/mol (CYP3A4, 4d6z) to –7.1 kcal/mol (CYP2B6, 4zv8), whereas mean E_{bind} for all human CYPs tested is ~9.3 kcal/mol for CYPs from *Homo sapiens*. The top 10 lowest E_{bind} for the ligand and the enzymes' structures are in the table 4.

Table 4

Top 12 E_{bind} values for CPF-bab-Z1 *in silico* interactions with structures of human CYPs

PDB code	CYP	E_{bind}, kcal/mol	Fe	N41
4d6zcA	CYP3A4	–12.3	Fe	N41-THR224
1w0ecA	CYP3A4	–12.1	Fe	N41-THR224, PRO227
5vcdcA	CYP3A4	–12.1	Fe	N41-ARG212, ALA370, HEM601
5vcccA	CYP3A4	–12	Fe	N41-ILE230
5vcccA	CYP3A4	–11.9	Fe	N41-ARG212, ALA305, THR309, HEM601
5vcdcA	CYP3A4	–11.9	–	N41-ILE223, ILE230
6bd6cA	CYP3A4	–11.9	–	N41-LEU210, LEU211
6bdicA	CYP3A4	–11.9	Fe	N41-ARG106
1tqncA	CYP3A4	–11.8	Fe	N41-ARG106, THR224
3juscA	CYP51	–11.8	–	N41-VAL143, ALA144, MET304
4d6zcA	CYP3A4	–11.8	–	N41-ARG106
5vcgcA	CYP3A4	–11.8	–	N41-ILE223, THR224, PRO227

The data obtained indicates that CPF-bab-Z1 could not be a potent inhibitor of human CYPs, with the exception of drug-metabolising liver CYP3A4. E_{bind} values have been found to be around –8.1, –9.0 and –11.0 for human CYP19, CYP17 and CYP51 structures (e. g. for structures with PDB codes 5jl6, 3ruk and 4uhi respectively).

In general, the new compound CPF-bab-Z1 was predicted to be able to bind effectively in the active sites of human cytochromes P450 CYP3A4 and, less effectively, CYP51. Its ability to bound in the cyclopropane-to-heme manner with some fungal CYP51 and in the azole-to-heme mode with some structures of CYP51 from *Leishmania* and CYP51 from *Trypanosoma* (see table 2, fig. 4) has also been revealed. This points out on a perspective of its practical testing as an inhibitor or a substrate for the corresponding enzymes.

Conclusion

Aiming to design new fluorescent inhibitor of P450s, we have synthesised the triazole-containing conjugate of ciprofloxacin, due to known ability of the anti-bacterial drug to fluorescent with blue light. The compound has been designed and synthesised. It was confirmed using HPLC and ESI-MS (m/z of the mass-to-charge ratio $[M + H]^+$ for $C_{28}H_{29}FN_7O_4^+$ is equal 546.23; found 546.20). Using *in silico* docking simulations we have tested the compound as a ligand for a number of cytochromes P450 of some pathogenic eukaryotes, baker yeast, mycobacteria and human. Our results have demonstrated that CPF-bab-Z1 is a potentially good ligand for CYP3A4 and CYP51 as well as for some fungal CYP51. Notably, the compound was predicted to be bound with some CYP51 in the cyclopropane-to-heme orientation. By contrast, for CYP51 from *Leishmania* (3l4d) and CYP51 from *Trypanosoma* (4g3j and 6fmo) the azole-to-heme mode like classical azole inhibitors of CYPs was predicted. The results offer interesting perspectives for testing of the compound as a new fluorescent P450 inhibitor of the enzymes mentioned.

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