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CONSERVATION OF DINITROANILINE/PHOSPHOROTHIOAMIDATE SITE OF α-TUBULIN IN *PLASMODIUM* SPECIES DISTRIBUTED IN INDIA

Aim. To evaluate polymorphism of dinitroaniline/phosphorothioamidate binding site in α-tubulin molecules from *Plasmodium* species (*P. falciparum, P. vivax, P. ovale*, and *P. malariae*) and strains discovered in India. ***Methods.*** Literature and database search. Bioinformatical comparison of protein sequences and structures. Multiple sequence alignment, phylogenetic profiling, protein structure modeling, etc. ***Results***. 14 complete sequences of α-tubulin from four *Plasmodium* species were selected from UniProtKB. Despite certain differences in complete sequences of α-tubulin molecules from different *Plasmodium* species and strains, sites of their interaction with dinitroaniline and phosphorothioamidate compounds were shown to be identical. ***Conclusions.*** Complete identity of dinitroaniline/phosphorothioamidate binding sites in all studied isotypes of α-tubulin molecules from *P. falciparum, P. vivax, P. ovale*, and *P. malariae* was confirmed. This suggests identity of the ligand-protein interaction mechanism and similar destabilizing effect of dinitroaniline and phosphorothioamidate compounds on microtubules of all *Plasmodium* species officially registered in India.

Keywords: malaria, *Plasmodium*, α-tubulin, dinitroanilines, phosphorothioamidates, binding site.

Plasmodium parasites are the main cause of global malaria infections with 300–500 million clinical cases and 1–3 million deaths per year [1]. It is known that malaria is caused at least by a few species of *Plasmodium* protozoan parasites: *P. falciparum, P. vivax*, *P. ovale*, and *P. malariae* [2]. The National Vector Borne Disease Control Program of India reported ~1.6 million cases and ~1100 malaria deaths in 2009 [3]. Historically, malaria in India was predominantly caused by *P. vivax*, accounting for 53% of the estimated cases. After the spread of drug-resistant *P. falciparum* in the 1990s, the prevalence of the two species remained equivalent. The burden of malaria in India is complex because of the highly variable malaria eco-epidemiological profiles, transmission factors, and the presence of multiple *Plasmodium* species and *Anopheles* vectors [4]. Now two major human malaria species in India are *P. falciparum* and *P. vivax*. *P. malariae* has been reported in the eastern India state of Orissa [5], while *P. ovale* appears to be extremely rare if not absent [3]. *P. falciparum* is most severe strain of the malaria species correlated with almost every malarial death (CDC - www.cdc.gov/malaria/about/disease.html). *P. falciparum* is strongly associated with severe disease syndrome known as cerebral malaria, which is associated with high mortality [2].

The control of malaria infection is hampered by many factors, including emerging drug resistance. It is a fact that many of existing now malaria therapeutics are increasingly ineffective and it is an urgent need in development of principally new therapeutic strategies and agents. During last ten years there has been a new growth of interest in tubulin as an important antiprotozoan target [6, 7] Several classes of microtubule (MT) inhibitors have demonstrate potent activity against malarial parasites in *in vivo*: vinblastine [8-10], dolastatin 10 [11], auristatins [12] and taxoids [13, 14]. Most of these agents have been demonstrated to disrupt or stabilize normal microtubular structures. Unfortunately, most all these compounds show toxicity to mammalian cells [15, 16] due to the interspecies conservation of tubulin [16]. *P. falciparum* and human α-tubulins share ~83% identity and β-tubulins ~87%. However it was found that human antimalarial drugs (e.g. sulfadiazine, sulfadoxine, pyrimethamine, cycloguanyl) were lethal for the model plant *Arabidopsis thaliana* at similar concentrations to market herbicides (glufosinate and glyphosate) [17]. Although MT inhibitors from anticancer programs have proved useful in probing MT function in parasites, such non-selective agents

have no prospects as antimalarial drugs [15, 16]. Later it was found that dinitroanilines (oryzalin, trifluralin, chloralin, pendimethalin) and phosphorothioamidates (aminprophos-methyl) destabilize MT and have overlapping sites in plant tubulin. It was proved that dinitroanilines and phosphorothioamidates bind plant α-tubulin with high affinity, inhibit tubulin assembly and disrupt MTs [18, 19]. Affected cells do not form a cell plate and may contain restitution and polyploidy nuclei [16].

It is very important that dinitroaniline/phosphorothioamidate site is absent in human tubulins [16, 20]. However, this site was found in some *Protozoa* [16]. This explains why some dinitroaniline derivatives with selectivity for parasite MTs are more than 800-fold effective on the parasite than on various human cell lines [21]. As a result of this relationship, studies have demonstrated that dinitroanilines and phosphorothioamidates in most cases active against plants are also active against *P. falciparum* and thus could act as antimalarial drug leads [17]. α-Tubulin appears to be an outstanding target for such parasitic diseases as leishmaniasis and trypanosomiasis, as the respective parasites also display altered or absent checkpoints [22, 23]. Phosphorothioamidates have similar effects on plants as the dinitroanilines and bind to the same molecular site. As phosphorothioamidates have a more than 100-fold higher solubility in aqueous solutions than dinitroanilines, these compounds are more promising candidates for modification than dinitroanilines, where biological studies indicated that maintaining high sufficient drug concentrations is difficult [24]. Therefore dinitroanilines and phosphorothioamidates can be considered as a priority group for the search of antimalarial agents.

The purpose of current study was to compare dinitroaniline/phosphorothioamidate binding site in α-tubulin molecules from *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) and strains, native for Indian region, and draw conclusions regarding the level of its polymorphism.

Materials and methods

Complete sequences of 14 α-tubulins from *P. falciparum* (PLAFA|PLAFK|PLAF7: A0A1D3KY74_PLAFA, A0A2Y9URV4_PLAFA, Q25790_PLAFA, TBA_PLAFK, Q6ZLZ9_PLAF7, Q8IFP3_PLAF7), *P. vivax* (A0A1G4GUY4_PLAVI, A0A1G4H9I2_PLAVI), *P. ovale* (A0A1C3KQ56_9APIC, A0A1A8VN48_9APIC, A0A1A8VPR2_9APIC, A0A1A8YLP3_9APIC), and *P. malariae* (A0A1A8VRI3_PLAMA, A0A1A8VSS4_PLAMA) were selected from UniProtKB [25].

The information on amino acid residues of dinitroaniline/phosphorothioamidate binding site in α-tubulin molecule and residues of known mutations upset ligand binding were based on the analysis of previously published results [20, 26-30].

Structural model of α-tubulin molecule from *P. falciparum* (TBA_PLAFK, UniProtKB: P14642) was built using protein structure homology-modelling server Swiss-Model [31] based on template RCSB Protein Data Bank (www.rcsb.org) [32] structures: 5UBQ (A) – α-tubulin from cilia of *Tetrahymena thermophila* (Cryo-EM structure) [33] and 2.5 E X-RAY structure 5KX5 (A) of α-tubulin from *Ovis aries* [34]. All visualizations and analysis of PDB-structures and constructed model of *Plasmodium* α-tubulin were performed using PyMOL v.1.5.0.5 software (www.pymol.org).

Multiple alignments of the complete amino acid sequences of α-tubulin molecules from different *Plasmodium* species and strains were performed with the ClustalX (V.2.0.10) (www.clustal.org) software using BLOSSUM weight matrices [35]. In the case of the comparison of small polypeptide fragments of the sites of interaction with dinitroaniline and phosphorothioamidate compounds, we applied direct comparison of the sequences in ClustalX with restriction of gaps [35]. All clustering was based on similarity of complete amino acid sequences and their fragments. Visualizations and analysis of dendrograms were performed with the MEGA7 software package (www.megasoftware.net/) [36].

Results and discussions

Initial analysis of information presented in the UniProtKB database revealed 17 α-tubulin sequences of four *Plasmodium* species distributed in India (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*). At the same time, only 14 of them are presented by complete sequences covering binding region of dinitroaniline and phosphorothioamidate compounds. From above mentioned sequences six of them belong to *P. falciparum* (isolates PLAFA, PLAFK/K1 and PLAF7/3D7), 2 – *P. vivax* (PLAVI), 4 – *P. ovale* (9APIC), and 2 – *P. malariae* (PLAMA). The subsequent alignment and reconstruction of the phylogenetic tree revealed variations in the complete α-tubulin sequences from different *Plasmodium* species, isolates and strains (Fig. 1). Thus, it was a probability of certain differences of dinitroaniline/phosphorothioamidate binding sites in α-tubulin molecules from different species and ecotypes.

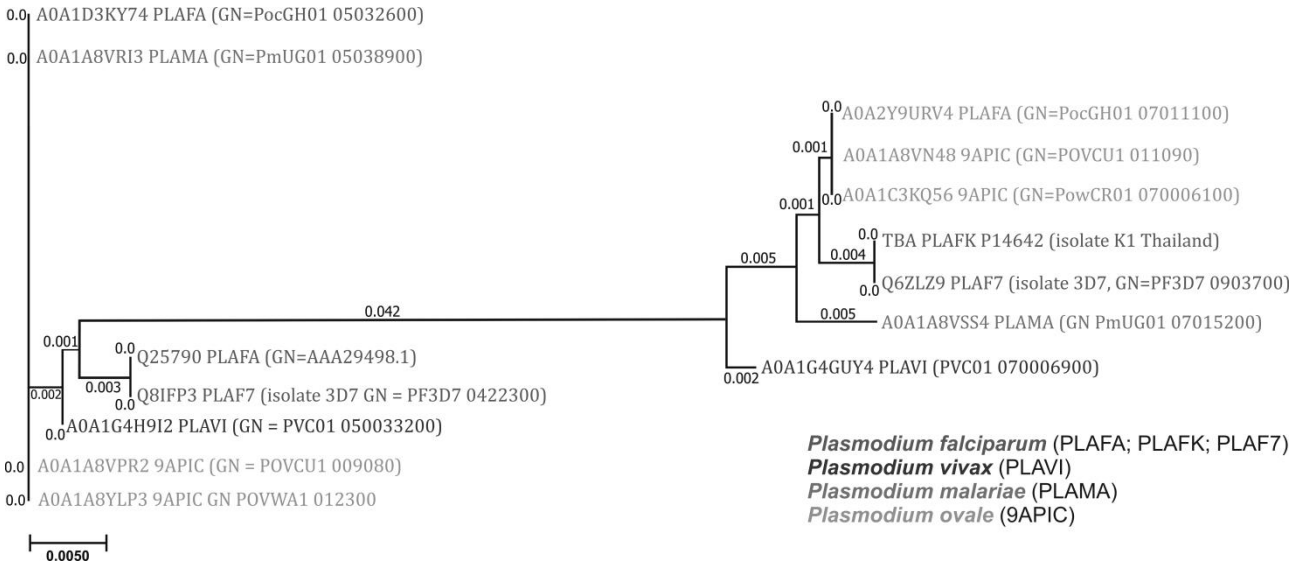

Fig. 1. Phylogenetic clustering of complete sequences of α-tubulin molecules from *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) and strains officially registered in India.

To reveal similarity of dinitroaniline/phosphorothioamidate binding site in α-tubulin molecules from different *Plasmodium* species, literature analysis was carried out and information on the amino acid composition and structure of this site among *Protozoa* (*P. falciparum* and *Toxoplasma gondii*) and higher plant *Eleusine indica* was summarized [20, 26-30]. Selecting functionally important amino acids, we used such criteria as direct participation in the binding of ligands, involvement in the structure of site, as well as amino acids, mutations on which cause resistance to dinitroaniline and phosphorothioamidate compounds (Table). Additionally, the amino acids described in the literature as related to the dinitroaniline / phosphorothioamidate binding site, were verified based on their structural position using 3D-model of α-tubulin from *P. falciparum* (TBA_PLAFK, UniProtKB: P14642) as control (Fig. 2).

Table. Amino acid residues of dinitroaniline/phosphorothioamidate binding site in α-tubulin and residues of known mutations upset ligand binding

Species	**Deposition in UniProtKB**	**Amino acids of binding site**	**Sourse of information**
Plasmodium falciparum	TBA_PLAFK (P14642)	Arg2, Gln133, Arg243, Asn249, Val250, Asp251, Val252, Thr253, Glu254	[27, 28]
Toxoplasma gondii	TBA_TOXGO (P10873)	Arg2, His28, Gln133, Thr239, Ala240, Arg243	[30]
		Arg2, Glu3, Val4, Trp21, Phe24, His28, Asp47, Arg64, Cys65, Thr239, Arg243, Phe244	[20]
		Val4, Ser6, Phe24, His28, Leu136, Ile235, Thr239, Arg243 (mutated in resistant parasites)	[29]
Eleusine indica	TBA1_ELEIN (O22347)	Arg2, Gln133, Arg243, Asn249, Val250, Asp251, Val252, Asn253, Glu254	[26, 27, 28]

Note. Italics mark amino acid residues, mutation of which causes resistance to dinitroaniline and phosphorothioamidate compounds.

Comparing known data, the results of structural modeling and alignment of 14 α-tubulin sequences from *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, the residues of dinitroaniline/phosphorothioamidate binding site were identified. Based on this information, the associated fragments were extracted from complete sequences. In addition to the above-mentioned amino acid residues, amino acids adjacent to them along the N- and C-directions of the polypeptide chain were also selected. The alignment of selected fragments reveals their complete identity, what was also confirmed by results of clustering (Fig. 3).

Thus, despite differences in complete amino acid sequences of α-tubulin from *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) and strains, distributed in India, their sites of interaction with dinitroanilines and phosphorothioamidates are completely identical. Considering inherent tubulins strong similarity of spatial structures [27, 37-39], it can be said with confidence that dinitroaniline and phosphorothioamidate compounds will have the similar effect on the MTs of all studied species and strains.

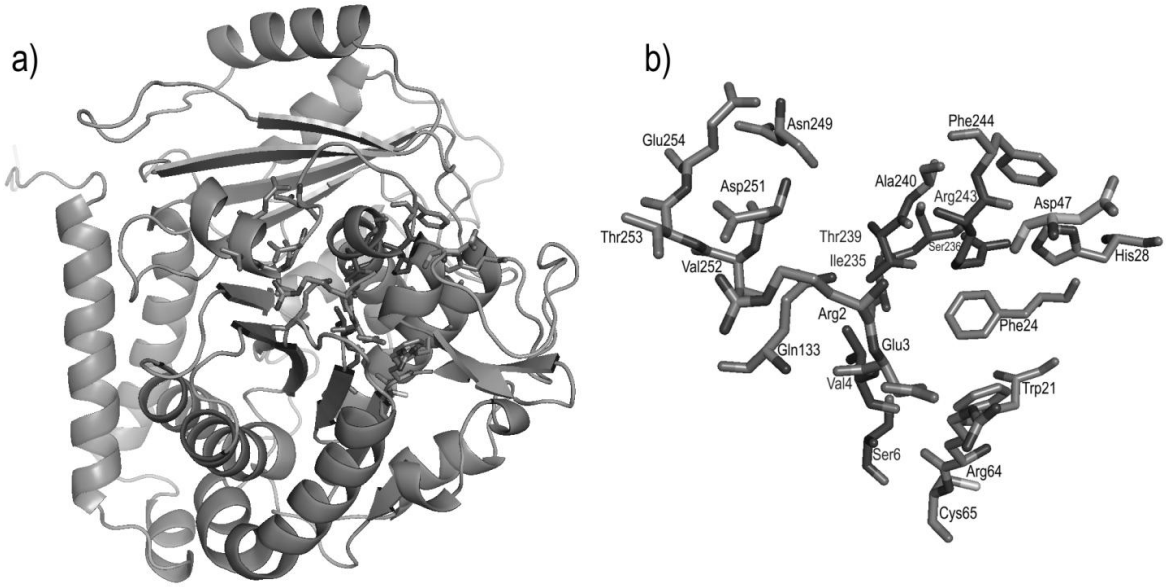

Fig. 2. Structural topology (a) of dinitroaniline and phosphorothioamidate binding site and its amino acids (b) in *P. falciparum* α-tubulin: Arg2, Glu3, Val4, Ser6, Trp21, Phe24, His28, Asp47, Arg64, Cys65, Gln133, Leu136, Ile235, Thr239, Ala240, Arg243, Phe244, Asn249, Val250, Asp251, Val252, Thr253 and Glu254. Red color (dark grey in monochrome representation) sticks mark amino acid residues, mutations of which cause resistance to dinitroanilines and phosphorothioamidates: Leu136, Ile235, Thr239 and Arg243.

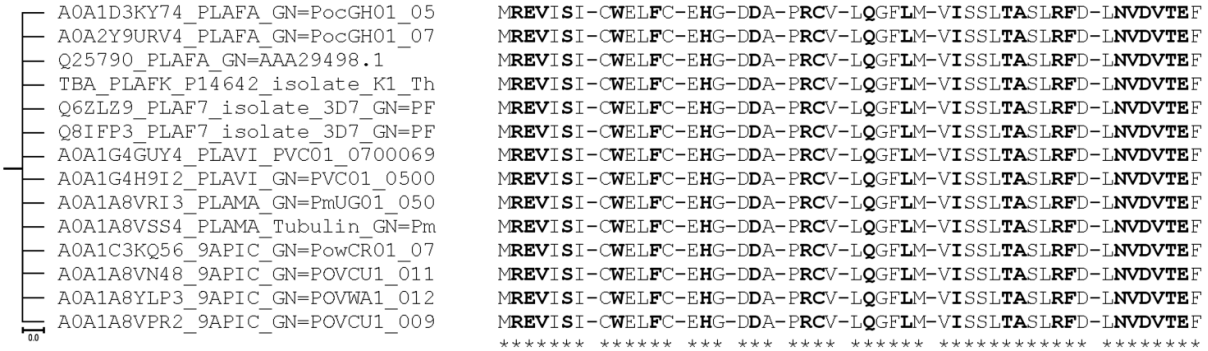

Fig. 3. Results of clustering (at the left) and alignment (on the right) of fragments, associated with dinitroaniline and phosphorothioamidate binding site in sequences of α-tubulins of studied strains of *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*, registered in India.

Conclusions

Complete identity of dinitroaniline/phosphorothioamidate binding sites in all studied isotypes of α-tubulin molecules from *P. falciparum, P. vivax*, *P. ovale*, and *P. malariae* was confirmed. Considering inherent tubulin molecules strong similarity of spatial structures, it can be concluded that individual dinitroaniline and phosphorothioamidate compounds will have the same effect on MTs of all *Plasmodium* species officially registered in India. Such conservatism makes it possible to simplify subsequent protocol of virtual screening and perform it against only one molecular target.

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КОНСЕРВАТИВНІСТЬ САЙТУ ЗВ'ЯЗУВАННЯ ДИНІТРОАНІЛІНІВ І ФОСФОРОТІОАМІДІВ МОЛЕКУЛАМИ α-ТУБУЛІНУ У РОЗПОВСЮДЖЕНИХ В ІНДІЇ ПРЕДСТАВНИКІВ РОДУ *PLASMODIUM*

Мета. З'ясувати ступінь поліморфізму сайту зв'язування динітроаніліну/фосфоротіоаміду на поверхні молекул α-тубуліну у різних штамів представників роду *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*), що зустрічаються на території Індії. ***Методи***. Аналіз літератури та баз даних. Біоінформатичне порівняння білкових послідовностей і структур. Множинні вирівнювання послідовностей, філогенетичне профілювання, моделювання просторової структури білків тощо. ***Результати***. З бази даних UniProtKB було відібрано 14 повних послідовностей α-тубуліну чотирьох видів роду *Plasmodium*. Встановлено, що незважаючи на певні відмінності повних послідовностей молекул α- тубуліну у різних видів і штамів *Plasmodium*, сайти їх взаємодії з похідними динітроаніліну і фосфоротіоаміду повністю ідентичні. ***Висновки***. Підтверджено повну ідентичність сайтів зв'язування динітроаніліну/фосфоротіоаміду у всіх досліджених ізотипів молекул α-тубуліну з *P. falciparum*, *P. vivax*, *P. ovale* та *P. malariae*. Це свідчить про ідентичність механізму ліганд-білкової взаємодії та схожий дестабілізуючий ефект похідних динітроаніліну і фосфоротіоаміду на мікротрубочки вищезазначених видів *Plasmodium*, що офіційно зареєстровані на території Індії.

Ключові слова: малярія, *Plasmodium*, α-tubulin, похідні динітроаніліну, похідні фосфоротіоаміду, сайт зв'язування.