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Inhibitory effect of piceatannol, a protein tyrosine kinase inhibitor, on asexual maturation of *Plasmodium falciparum*

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This report describes the effect of piceatannol (3,4,3',5'-tetrahydroxy *trans* stilbene), a plant secondary natural product, on protein tyrosine kinase (PTK) activity in different stages of *P. falciparum* grown *in vitro*. Piceatannol inhibited PTK activity in trophozoites and schizonts suggesting that PTK may be important in the initial asexual maturation of the parasite. Inhibition of PTK activity by piceatannol may thus provide new insights into more specific tools for chemotherapeutic interventions for *P. falciparum*.

Protein tyrosine kinases (PTKs) are the principal signal enzymes enabling cell to cell communication, growth regulation and differentiation[1]. PTKs are therefore potentially important drug targets due to their role as positive regulators of cell proliferation[2,3]. Growing resistance of *Plasmodium falciparum* to chloroquine[4,5] and other antimalarials has led to the search of other antimalarials including natural plant products[6,7]. In this context, therefore, a search for naturally occurring plant products with inhibitory activity towards PTK could yield new antimalarials for the study of protein phosphorylation leading to new drug designs and more potent inhibitors. We report here, that one such compound piceatannol, an antileukemic principle in the seeds of *Euphoria lagascae*, has been found to be inhibitory to *P. falciparum* protein tyrosine kinase during the asexual maturation of the parasite *in vitro*. The results suggest that the PTK activity may be of use as chemotherapeutic drug target and new drug development in *P. falciparum* resistant malaria.

Plasmodium falciparum, FSJ-M strain was maintained in continuous culture according to the method of Trager and Jensen[8]. The cultures were synchronized with aqueous D-sorbitol treatment and synchronized cultures with either ring-, trophozoite-, or schizont-infected erythrocytes were further concentrated by Percoll density gradient centrifugation at 1800*g* for 30 min at 4° C(ref. 9). The parasitaemia and the stage of parasitic development was monitored by microscopic observation of JSB-stained blood smears. Infected and uninfected erythrocytes were separated by centrifugation (700*g*, 10 min, 4°C) and lysed with PBS, *p*H 7.0. Released parasites were washed three times in PBS and were concentrated by centrifugation and sonicated and centrifuged at 1,50,000 *g* for 15 min at 4°C for cytosol and parasite membranes preparation in the Beckman L8-M ultracentrifuge. The parasite cytosol and membranes were stored frozen in aliquots in 20 m*M* Tris buffer, *p*H 7.0 containing a mixture of protease inhibitors (30 µg/ml soyabean trypsin inhibitor, 1m*M* PMSF, 5 m*M* iodoacetamide, 5 µg/ml leupeptin and pepstatin A). The Protein tyrosine kinase activity was measured according to the method of Rijksen *et al.*[10] using a non-radioactive method with some modifications. Briefly, the standard Kinase assay mixture contained buffer (50 m*M* Tris/HCl, *p*H 7.5, 20 m*M* Mg-acetate, 5 m*M* NaF, 0.2 m*M* EDTA, 0.8 m*M* EGTA, 1.0 m*M* dithiothreitol, 30 µ*M* Na_4 VO4, 300 µ*M* ATP), peptide substrate (PKS2) 1 µ*M* (biotin-EGPWLEEEEEAYGWMDF-amide,) ATP (1m*M*), Mg^{+2} (10 m*M*) and the enzyme sample (10 µg) . Minimal reaction volume was 50 µl. The assay system was incubated at 37°C for 60 min. After the enzyme reaction was quenched by the specific inhibitor (120 m*M*, EDTA *p*H 7.2), phosphorylated and dephosphorylated substrate was immobilized by binding to a streptavidin coated microtitre plate, and the reaction mixture was washed out. The fraction of the phosphorylated substrate was determined immunochemically with ABTS (a registered

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Table 1—Protein tyrosine kinase activity in cytosolic and membrane fractions in different maturation stages of *Plasmodium falciparum* and its inhibition by piceatannol

[Values expressed as pmole phosphate/ mg protein/min, are mean ±SD of 5 separate experiments]

	Protein tyrosine kinase activity			
	Cytosol		Membrane	
Stage	Status	Piceatannol	Status	Piceatannol
Ring	4.4 ± 0.6	3.9 ± 0.4	5.0 ± 0.5	2.0 ± 0.3
Trophozoite	6.5 ± 0.8	3.8 ± 0.4	5.4 ± 0.6	2.8 ± 0.3
Schizont	6.9 ± 0.7	4.7 ± 0.6	6.0 ± 0.5	3.7 ± 0.6

trademark of Boehringer Mannhein,GmbH) via a highly specific antiphosphotyrosine antibody, directly conjugated to peroxidase. The absorbance was measured at 405 nm (reference wavelength approx 490nm) using a microtitre plate reader(ELISA reader).The enzyme activity was expressed as pmoles of phosphate incorporated into substrate (PKS2) per mg protein per minute. The protein concentration in the samples was measured according to the method of Lowry *et al.* [11].

The association between cellular transformation, maturation and altered protein kinase activity, particularly on the tyrosine residues, has been well established by several lines of evidence in a number of transformed systems[2,12]. These alterations could either be in the kinase substrates or in the activity of protein kinases. The relative protein tyrosine kinase activity in the cytosol and the membrane extracts varied from one stage to the other in *P. falciparum* (Table 1). As the parasite matures from the ring stage to the schizont stage the protein tyrosine kinase activity was found to be increased in cytosol and also in the membranes.

However, since we were interested in inhibition of the protein tyrosine kinases , we tested the ability of the piceatannol to inhibit the activity of PTK in *P. falciparum*, which have been found to overexpress the membrane bound PTKs known to be p56 *lck* [13]. Piceatannol is a bioactive stilbene that has been isolated from the plant sources (Fig. 1). A synthetic sample of the piceatannol was tested for its ability to inhibit the activity of cytosolic protein tyrosine kinase isolated from the *P. falciparum*. The phosphorylation of the substrate(biotin peptide amide) on the tyrosine residues was inhibited by increasing concentrations of piceatannol and maximum inhibition was observed at a concentration of 750 µg/ml or 3m*M* piceatannol (Fig. 1). In all further inhibition assays 3 m*M* piceatannol was added. The mode of action of

Table 2—Multiple comparison of the activity of protein tyrosine kinase between pairs of various maturation stages of *Plasmodium falciparum*

Comparison	Protein tyrosine kinase activity	
	Cytosol	Membranes
Ring stage versus		
Trophozoite	S	S
Schizont	S	S
Gametocyte	NS	NS
Trophzoite versus		
Schizont	S	S
Gametocyte	NS	NS
Schizont versus		
Gametocyte	NS	NS

NS = Non significant; S = Significant

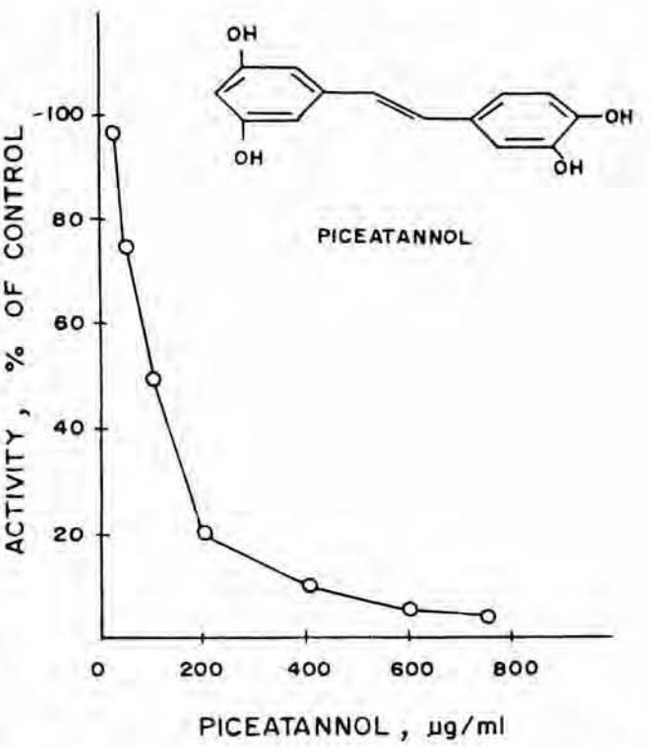

Fig. 1—Inhibition of cytosolic protein tyrosine kinase activity by increasing concentrations of piceatannol.

piceatannol is very much similar to that observed for the synthetic analogs like tryphostins[14,15] and /or erbstatin[16] and derivatives of 4-hydroxycinnamamide[17] which all share a common structural similarity with tyrosine. Therefore piceatannol would show little inhibitory activity towards protein kinases with a substrate specificity for serine and threonine. Our results show that piceatannol can inhibit the activity of these kinases in all the asexual parasite stages (Table 1). Multiple comparison analysis among pairs demonstrate that the activity in gametocytes was not significantly different as compared to ring, trophozoite and schizont stages of the parasite maturation (Table 2). The present findings thus show that protein tyrosine kinase activity may therefore be required only during the asexual development of the parasite and probably it does not have any role in the sexual stages.

An effective inhibitor of the protein kinases will be one that could penetrate the lipid bilayers to inhibit the activity of intracellular tyrosine kinases. Certain tyrphostins, can inhibit the tyrosine kinases of the EGF receptors in cultured cells. Piceatannol has been found to inhibit the activity of protein tyrosine kinases both in the cytosol and in the membrane. The identification of piceatannol as an inhibitor provides important information regarding the types of the compounds that are capable of interacting at the protein substrate binding sites. Our data also indicate that the PTK activity is differentially expressed during the parasite development and may have critical roles in the regulation of cellular events and new drug development in the *P. falciparum* resistance malaria. Efforts are underway to isolate phosphotyrosine containing protein p56 *lck* and inhibit the phosphorylation of this protein *in vitro* and use this compound as inhibitor of the maturation of the malarial parasite.

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