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Antimalarial potential of xestoquinone, a protein kinase inhibitor isolated from a Vanuatu marine sponge *Xestospongia* sp.

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Abstract—As part of our search for new antimalarial drugs, we have screened for inhibitors of Pfnek-1, a protein kinase of *Plasmodium falciparum*, in south Pacific marine sponges. On the basis of a preliminary screening, the ethanolic crude extract of a new species of *Xestospongia* collected in Vanuatu was selected for its promising activity. A bioassay-guided fractionation led us to isolate xestoquinone which inhibits Pfnek-1 with an IC_{50} around 1 μM. Among a small panel of plasmodial protein kinases, xestoquinone showed modest protein kinase inhibitory activity toward PfPK5 and no activity toward PfPK7 and PfGSK-3. Xestoquinone showed in vitro antiplasmodial activity against a FCB1 *P. falciparum* strain with an IC_{50} of 3 μM and a weak selectivity index (SI 7). Xestoquinone exhibited a weak in vivo activity at 5 mg/kg in *Plasmodium berghei* NK65 infected mice and was toxic at higher doses.

1. Introduction

Very little is known about the molecular mechanisms controlling cell growth and development in malaria parasites. A major hindrance to progress in this field arises from the difficulties in manipulating the parasite genome: although transient transfection experiments and knock-out of genes which are not essential to the asexual erythrocytic cycle are now possible, no systems are yet available to induce expression of transfected genes or to inactivate genes that are essential for the completion of asexual growth. However, significant information can be gained by characterizing gene products at the biochemical level. Among the estimated 6000 genes of *Plasmodium falciparum*, the 85 gene products related to eukaryotic protein kinases constitute potential targets.[1,2] If a given enzyme can be shown to be essential for parasite development, inhibition assays can be developed to screen chemical libraries, and identified hit compounds subsequently be tested for their effect on the development of the parasite in vitro.

As part of our search for new drugs against malaria, we have searched for inhibitors of Pfnek-1, a never-in-mitosis/*Aspergillus* (NIMA)-related protein kinase of *P. falciparum*,[3] in extracts from South Pacific marine sponges.[4] On the basis of promising properties in a preliminary screen on Pfnek-1 activity, the ethanolic crude extract

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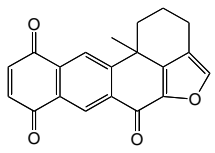
of a new species of *Xestospongia* collected in Vanuatu was selected. Marine sponges of the genus *Xestospongia* (Haplosclerida, Petrosiidae) have proven to be a rich source of diverse secondary bioactive metabolites including alkaloids (xestospongins/araguspongines, aaptamines, manzamines, ingenamines, and renieramycins), polycyclic quinones and hydroquinones, polyacetylenic derivatives, aminoalcohols, heterocyclic compounds, and original sterols (aragusterol). Some of these compounds exhibit significant cytotoxic, antimicrobial, antiplasmodial or vasodilatory activities.[5] Some protein tyrosine kinase inhibitors were also isolated from this genus, such as polycyclic quinones and hydroquinones of the halenaquinone class.[6,7]

A bioassay-guided fractionation based on a Pfnek-1 inhibition assay led us to isolate two previously described molecules: xestoquinone (**1**) and halenaquinone (**2**). The antiplasmodial activity of xestoquinone, the most potent and the most abundant Pfnek-1 inhibitor, was studied in vitro against a *P. falciparum* strain and in vivo in *Plasmodium berghei* NK65 infected mice.



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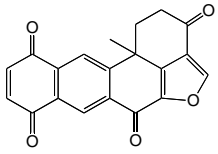

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2. Results

Xestoquinone inhibited Pfnek-1 activity with an average IC_{50} value of 1.1 μM, whereas halenaquinone exhibited a weaker activity (IC_{50} around 3 μM). Xestoquinone showed modest protein kinase inhibitory activity toward PfPK5 and no activity toward PfPK7 and PfGSK-3. Toward kinases from higher eukaryotes, xestoquinone expressed modest inhibitory activity against Erk2 and no activity against GSK-3α/β, CDK1/cyclin B, CDK5/p25, and PKA (Table 1).

Xestoquinone showed a moderate antiplasmodial activity with an IC_{50} of 3 μM. In order to assess whether the observed activities are specific or due to a general toxic effects, we calculated an experimental selectivity index (SI) defined as the ratio of cytotoxicity to antiplasmodial activity. Xestoquinone exhibited a cytotoxic activity on MCF7 cells with an IC_{50} of 20 μM, therefore the SI of xestoquinone was calculated as 7.

The amount of 20 mg/kg/4 days was found to be lethal at the second injection for the mouse infected by *P. berghei* NK65. On the other hand, the mice could stand a treatment with 5 mg/kg/4 days. The treatment was beneficial over the 10 days following the test since 50% of the mice survived, whereas 9 out 10 untreated mice died in the interval. In comparison, all the mice treated with 10 mg/kg chloroquine survived. By the end of the treatment, the parasitemia was inhibited by 100% with chloroquine, whereas it was inhibited only by 47% in the mice treated with the xestoquinone. Four days after the end of the treatment, the action of chloroquine was always present with 100% of inhibition of the parasitemia, whereas, in the remaining mice, inhibition by the xestoquinone was no longer significant.

Table 1. Effects of xestoquinone on the activity of several protein kinases

Protein kinase	IC_{50} (μM)
Pfnek-1[a]	1.10
PfPK5	17.00
PfPK7	>100.00
PfGSK-3	160.00
GSK-3α/β	140.00
CDK1/cyclin B	60.00
CDK5/p25	>100.00
Erk2	11.00
PKA	>100.00
$pp60^{v\text{-}src}$[b]	60.00
EGFR[b]	60.00

[a] Data obtained for Pfnek-1 results from experimentations carried out on different enzymes prepared in three independent laboratories.
[b] Data from Lee et al.[6] and Alvi et al.[7]

Xestoquinone was inactive at 2.5 mg/mL in the ferriprotoporphyrin IX (FP) biomineralization assay.

3. Discussion

Kinase inhibitors are widely employed as biological reagents and as leads for drug design, but their use is often complicated by their lack of specificity. Therefore, it was important to evaluate the selectivity of the protein kinase inhibitory activity of xestoquinone against a panel of *P. falciparum* protein kinases including PfPK7, PfPK5, PfGSK-3, in addition to Pfnek-1, as well as kinases from higher eukaryotes including GSK-3α/β (glycogen synthase kinase), CDK1/cyclin B and CDK5/p25 (cyclin-dependent kinase),[8,9] Erk2 (extracellular signal-regulated kinase),[10] and PKA (protein kinase A)[11] (Table 1).

- PfPK7 is an atypical enzyme distantly related to the MAPKK family (mitogen-activated protein kinase kinase).[12]
- PfPK5 is a member of the CDK family (cyclin-dependent kinase) which regulates the cell division cycle, apoptosis, transcription and differentiation in addition to functions in the nervous system.[13,14]
- PfGSK-3 is one of three *P. falciparum* kinases related to GSK-3, a ubiquitous kinase involved in Wnt signaling, glycogen metabolism, development, differentiation, cell cycle, and apoptosis.[15]
- Pfnek-1 is a serine/threonine malarial kinase. This enzyme displays maximal homology to the never-in-mitosis/*Aspergillus* (NIMA)/NIMA-like kinase (Nek) family, whose members are involved in eukaryotic cell division processes.[3]

Although, xestoquinone appears relatively selective, based on current data, more work is necessary to identify the scope of targets xestoquinone interacts with.

Compared to oxindole-based compound activity, xestoquinone was not the best inhibitor of PfPK5 with an IC_{50} of 17 μM versus 1.5 μM.[16]

The protein tyrosine kinase inhibitory activity of xestoquinone and halenaquinone has been reported previously.[6,7] More recently, Cao et al. showed that halenaquinone and xestoquinone at a lesser degree inhibited recombinant human Cdc25B phosphatase (IC_{50} values of 0.7 and 12 μM, respectively).[17] Halenaquinone was effective against both the $pp60^{v\text{-}src}$ and epidermal growth factor (EGF) receptor PTKs with IC_{50} values of 1.5 and 19 μM, respectively. In contrast, xestoquinone displayed a weak activity on these two kinases with IC_{50} values of 60 μM. Unlike xestoquinone, halenaquinone is also an inhibitor of phosphatidylinositol 3-kinases, enzymes which phosphorylate phosphatidylinositol 4,5-diphosphate (PIP2) to form phosphatidylinositol 3,4,5-triphosphate (IP_3).[18] Xestoquinone also displays activity against other enzymatic systems. It is a powerful cardiotonic agent with inotropic action which directly activates actomyosin ATPase activity in cardiac and skeletal myofibrils.[19–21] Xestoquinone also inhibits topoisomerases I and II,[22] and was also reported as cytotoxic against KB cells and L1210 cell lines.[20] In contrast, Schmitz and Bloor showed that this product was inactive against PS cells.[23] Following an evaluation in the National Cancer Institute (Development Therapeutics Program) in vitro multicell line screen, xestoquinone and halenaquinone were potent growth inhibitors but ineffective as cytotoxins in assays against different types of tumor cells.[7]

The antiplasmodial activity of xestoquinone is moderate. In comparison, ilimaquinone, the only other known marine antiplasmodial quinone, isolated from the Australian marine sponge *Dactylospongia elegans* Thiele, 1899, exhibited an in vitro activity against K1 and NF54 *P. falciparum* clones at 5 and 2.6 μM, respectively.[24]

Xestoquinone exhibits a slight antimalarial activity. Without explanation on the exact role of Pfnek-1 in *Plasmodium* biochemistry, we cannot conclude if this in vivo activity depends on the inhibition of protein kinase activity. The mode of action of this compound appears to be unrelated to the ferriprotoporphyrin IX (FP) biomineralization process. FP biomineralization is a *Plasmodium* specific process in which FP derived from the digestion of ingested hemoglobin is converted to hemozoin (β-hematin). The inhibition of biomineralization has been shown to be valuable for the detection of compounds with potential antimalarial activity.[25] Compounds with antiplasmodial activity that correlates well with the ability to inhibit FP degradation include terpene isonitriles, certain bisquinolines, FP analogs, xanthones, 8-aminoquinolines, and other synthetic antimalarial drugs.[26]

4. Conclusion

In summary, we believe that xestoquinone constitutes a promising scaffold from which more potent and more selective inhibitors of *Plasmodium* kinases could be designed. Biotinylated derivatives of xestoquinone could be used as an affinity chromatography matrix to allow the purification and identification of its targets in *Plasmodium* as well as in its host tissues.[27]

5. Experimental

5.1. General

A sponge of the genus *Xestospongia* (order Haplosclerida, family Petrosiidae) was collected by scuba diving at 46 m depth at Malvoror reef, near Santo Island (Vanuatu). A voucher specimen, Voucher No. G318469, is deposited at the Queensland Museum, Brisbane, Australia. The FCB1 strain of *P. falciparum* was kindly provided by Dr. A. Valentin, Laboratory of Parasitology, Faculty of Pharmacy, Montpellier, France.

Solvents were purchased from Ajax, Australia, and distilled before use, except for the HPLC grade acetonitrile and methanol; Biochemical reagents were purchased from Sigma–Aldrich and Cambrex. The radioactivity, γ-[^{33}P]ATP, was purchased from Perkin Elmer, France.

HPLC was performed on Waters apparatus, using an hypersil 100 C_{18} column (Waters 510 pumps; Waters 996 Photodiode Array Detector). NMR spectra were recorded on Bruker AC 250 or Bruker Avance 300 spectrometers. Mass spectra were recorded on a Nermag R 10-10 spectrometer. Radioactivity was measured using a liquid scintillation analyzer Packard Tri-carb 1600TR and Packard Ultima Gold MV scintillation cocktail. Antiplasmodial and cytotoxic activities were recorded on a Polarstar BMG microplate reader spectrophotometer. The heme polymerization was evaluated with a micro-ELISA reader (Titertek Multiskan MCC/340).

5.2. Extraction, isolation and spectroscopic data

The freeze-dried sponge sample was extracted twice overnight with fresh 95% EtOH at room temperature. The pooled ethanolic extracts were filtered and evaporated. The residue was partitioned between CH_2Cl_2 and H_2O and then AcOEt and H_2O. After evaporation, the AcOEt solubles were then subjected to Si-gel column chromatography (Merck Silica gel 60, 0.040–0.063 mm) using increasing concentration of MeOH in CH_2Cl_2. A fraction obtained with CH_2Cl_2–MeOH 98/2 (v/v) gave xestoquinone and halenaquinone enriched mixture. Xestoquinone (t_R = 18 min) and halenaquinone (t_R = 23 min) were purified by reversed-phase HPLC (hypersil 100 C_{18} column) with water–acetonitrile gradient elution (20/80–0/100 (v/v) for 30 min, flow rate: 1 mL/min, wavelength: 296 nm). Their spectroscopic properties were consistent with previously reported data.[19]

Xestoquinone: yellow powder; EIMS: 303, 318 (M^+); DCIMS (NH3): 303, 319 (MH^+), 336 (MNH_4^+); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.53 (s, 3H), 1.75 (ddd, J = 4.7 Hz, $J = J$ = 13.0 Hz, 1H), 2.16–2.27 (m, 2H),

2.57 (ddd, $J = J = 3.6$ Hz, $J = 12.8$ Hz, 1H), 2.64 (dddd, $J = 1.5$ Hz, $J = 8.4$ Hz, $J = 9.8$ Hz, $J = 17.1$ Hz, 1H), 2.88 (dddd, $J = 1.5$ Hz, $J = 2.5$ Hz, $J = 8.0$ Hz, $J = 17.1$ Hz, 1H), 7.02 (s, 2H), 7.54 (t, $J = 1.5$ Hz, 1H), 8.23 (s, 1H), 9.03 (s, 1H); ^{13}C NMR (300 MHz, $CDCl_3$): δ 16.9, 18.4, 31.2, 32.6, 37.4, 121.5, 123.2, 127.0, 130.3, 133.2, 137.9, 138.7, 139.4, 144.0, 145.0, 147.3, 156.2, 170.3, 183.9, 184.7.

Halenaquinone: yellow oil; EIMS: 317, 332 (M^+); DCIMS (NH_3): 333 (MH^+), 350 (MNH_4^+); ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.69 (s, 3H), 2.30 (dd, $J = 5.2$ Hz, $J = 13.2$ Hz, 1H), 2.81–3.05 (m, 3H), 7.09 (s, 2H), 8.28 (s, 2H), 9.09 (s, 1H).

5.3. Bioguiding assay

The chemical fractionation was bioguided with the following protein kinase assay: Recombinant Pfnek-1 was purified from an ampicillin-resistant bacteria *Escherichia coli* (BL21 strain) expressing a fusion protein containing a glutathione-*S*-transferase moiety (used for enzyme purification by affinity chromatography on a glutathione resin) and the catalytic domain of Pfnek-1.[3] The protein kinase activity was estimated by measuring ^{33}P incorporation into β-casein, using γ-[^{33}P]ATP as a phosphate donor. Briefly, test compounds were solubilized in DMSO and incubated in 20 mM Trizma, pH 7.5, containing 20 mM $MgCl_2$, 10 mM NaF, and 10 μM ATP. β-Casein (3 mg/mL) and γ-[^{33}P]ATP were added prior to the addition of 1 μg GST-Pfnek-1, which initiated the kinase reaction. Approximately 5 μCi of γ-[^{33}P]ATP was used per reaction. After incubation at 30 °C for 30 min, each solution was transferred to 2.5 × 2.5 cm piece of Whatman P81 phosphocellulose paper, and, 20 s later, the filters were washed four times (15 min each time) in a solution of 10 mL phosphoric acid/liter of water. The acid-precipitable radioactivity was counted 2 h after the introduction of dry papers in the scintillation fluid. The IC_{50}, defined as the concentration of compounds which inhibits 50% of enzyme activity, was estimated from the dose–response curves.

5.4. Protein kinase selectivity study

The kinase inhibition selectivity of xestoquinone was evaluated against a panel of *P. falciparum* protein kinases including PfPK7,[12] PfPK5,[13,14] and PfGSK-3,[15] in addition to Pfnek-1. These proteins were expressed and assayed as previously described. Controls were performed with appropriate dilutions of dimethylsulfoxide.

GSK-3α/β was purified from porcine brain by affinity chromatography on immobilized axin.[28] It was assayed, following a 1/100 dilution in 1 mg bovine serum albumin/mL 10 mM D-L-dithiothreitol (DTT), with 5 μL of 40 μM GS-1 peptide (YRRAAVPPSPSLSRHSSPHQ-SpEDEEE) as a substrate, in buffer A (10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 25 mM Tris–HCl, pH 7.5, and 50 μg heparin/mL), in the presence of 15 μM γ-[^{33}P]ATP (3000 Ci/mmol; 1 mCi/ml) in a final volume of 30 μL. After 30 min incubation at 30 °C, 25 μL aliquots of supernatant were spotted onto 2.5 × 3 cm pieces of Whatman P81 phosphocellulose paper, and, 20 s later, the filters were washed five times (for at least 5 min each time) in a solution of 10 mL phosphoric acid/liter of water. The wet filters were counted in the presence of 1 mL ACS (Amersham) scintillation fluid.

5.5. In vitro antiplasmodial bioassay

The antiplasmodial activity of xestoquinone was estimated in vitro against the chloroquine-resistant FCB1 strain of *P. falciparum* using the lactate dehydrogenase (PfLDH) micromethod.[29] Erythrocytes infected with *P. falciparum*, from cultures obtained using the method of Trager and Jensen,[30] were re-suspended in complete culture medium at a hematocrit of 1.5%. The suspension was distributed in 96-well microtiter plates (200 μL per well). Compounds were tested in triplicate in 1% parasitemia cultures mostly at the ring stage. For each assay, a parasite culture was incubated with the compound for 48 h in 5% CO_2 at 95% relative humidity and frozen until the biochemical assay could be run. A 20 μL sub-sample from each well was mixed with 100 μL of a substrate solution containing 2 mg of lithium L-lactate (Sigma), 0.55 mg TRIS (Sigma), and 0.37 mg of 3-acetylpyridine adenine dinucleotide (APAD; Sigma) per mL, in the well of another microtiter plate. After incubation for 30 min, 25 μL of a mixture of nitro blue tetrazolium (NBT) (1.6 mg/mL; Sigma) and phenazine ethosulfate (PES) (0.1 mg/mL; Sigma) was added to each well. Formation of the reduced form of APAD was measured at $\lambda = 650$ nm. IC_{50} values were determined graphically in a inhibition percent versus concentration curve.

5.6. Cytotoxicity assay

5.6.1. General procedures. Human breast adenocarcinoma (MCF-7) cells were cultured in DMEM culture medium containing 2 mM L-glutamine (Cambrex) supplemented with 10% fetal calf serum (FCS) (Cambrex) and incubated under standard conditions (37 °C, 5% CO_2). All experiments were carried out using cells in the exponential phase of growth. Cells were trypsinized, resuspended in DMEM containing 5% FCS and seeded (20,000 cells/well) in 96-well plates. After 24 h, the medium was replaced by fresh medium containing the compound. Fresh complete medium without drugs was used for controls. At the end of the treatment, cell viability was evaluated measuring the activity of the mitochondrial enzyme succinate dehydrogenase. This test used 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide sodium salt (XTT sodium salt) (Sigma) as substrate that was converted to a formazan product, which was detected spectrophotometrically at 450 nm.

5.6.2. Screening test. Cells were treated for 48 h with concentrations ranging from 1 to 100 μg/mL of the compound as shown by Tabbi et al.[31] After 48 h of compound exposure, the medium was replaced by 50 μL of an XTT solution (0.5 mg/mL), and cells were incubated for 180 min. The reaction was stopped by addition of a sodium dodecyl sulfate solution (10% in water). IC_{50} values were determined graphically from the dose–response curves.

5.7. In vivo bioassay

The in vivo antimalarial activity of xestoquinone was determined by the classical 4-day suppressive test of Peters against *P. berghei* Vincke and Lips 1948 (NK65).[32] Swiss female mice (Dépré, France), mean body weight 20 ± 2 g, were infected with 10^7 parasitized cells in 0.9% saline, on day 0. Groups of 10 mice were treated intraperitoneally from day 0 to day 3 with increasing doses of xestoquinone ranging from 2.5 to 20 mg/kg. The suppressive effect was estimated on day 4 by examining Giemsa-stained thin blood smears made from the tail of the mice treated with quinoxalines and compared with a control group of mice injected with saline. The stained thin blood smears were examined under 1000× magnification, counting at least 9000 erythrocytes, and the percentage of parasitized red blood cells was calculated for each concentration and compared to that observed in untreated animals. ED_{50} values were computed by comparing the parasitemias present in infected controls with those of test animals.

5.8. Heme polymerization assay

Xestoquinone was dissolved in a CH_2Cl_2–DMSO (1/16) solution. The ability of the xestoquinone to inhibit ferriprotoporphyrin IX (FPIX) biocrystallization was assessed by following the protocols previously reported by our group.[33] A mixture containing 50 μL of product solutions at 10 mg/mL (2.5 mg/mL final concentration in the well) or 50 μL of solvent (CH_2Cl_2–DMSO (1/16) for control), 50 μL of 0.5 mg/mL of hemin chloride (Sigma) freshly dissolved in DMSO, and 100 μL of 0.5 M sodium acetate buffer, pH 4.4, was incubated in a normal nonsterile flat-bottomed 96-well plate at 37 °C for 20 h. The solutions were added in the following order: first the hemin chloride, second the buffer, and finally the solvent or product. After incubation, the plate was centrifuged at 1600*g* for 5 min. The supernatant was discarded by vigorously flipping of the plate upside down twice. The remaining pellet was resuspended with 200 μL DMSO to remove unreacted FPIX. The plate was then centrifuged once again and the supernatant eliminated as described above. The pellet consisting of β-hematin was dissolved in 200 μL of 0.1 M NaOH for spectroscopic quantification at 405 nm. The data were expressed as the percentage of inhibition of FPIX biocrystallization calculated by the equation:

$$\%\ \text{inhibition} = 100 \times [(\text{OD control} - \text{OD drug})/(\text{OD control})].$$

Xestoquinone was tested in triplicate. Quinine hydrochloride in DMSO (IC_{50}: 0.04 mg/mL) was used as positive control.

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