Xanthurenic Acid Induces Gametogenesis in Plasmodium, the Malaria Parasite

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A small, heat stable chromophore extracted from mosquitoes has recently been implicated as the signal that induces mating of Plasmodium, the malaria parasite. We have used high resolution electrospray mass spectrometry to determine that this gamete activation factor (GAF) has a m/z = 205.0450, suggesting a molecular species composition of C_{10}H_{6}NO_{3}. Xanthurenic acid (XA), a product of tryptophan catabolism, was determined to have an elemental composition, ultraviolet absorbance maxima, and mass spectrum consistent with those characteristics of GAF. XA activated gametogenesis of Plasmodium gallinaceum and P. falciparum in vitro at concentrations lower than 0.5 μM in saline buffered to pH 7.4. A structural analog of XA, kynurenic acid (C_{10}H_{6}NO_{3}), also activated gametogenesis but only at higher concentrations and with less effect. We propose that XA is GAF. This is the first evidence that XA has induction activity.

Species of Plasmodium, the parasitic protozoa that cause malaria, must undergo one sexual recombination in each transmission cycle. Although the mature sexual stages, or gametocytes, are present in the blood of infected vertebrate hosts, mating only occurs in the gut lumen of vector mosquitoes. This process of gametogenesis begins immediately after infected blood reaches the gut and is completed within about 20 min. During that brief time the parasite dissolves two erythrocyte membranes surrounding it and, in the case of the male gamete, completes a profound nuclear reorganization that results in the formation of several flagellar, free swimming gametes (1). It has been postulated that the signal that activates these dramatic events is in the mosquito gut (2). We recently confirmed the existence of such a signal and demonstrated that it is a heat stable chromophore of M_e ≈ 205 (3). We report here the results of further chemical analyses that demonstrate that gamete activation factor (GAF) is xanthurenic acid (XA), a tryptophan metabolite. Although XA is widely present in both vertebrates and invertebrates, this is the first evidence that it has inductive activity. It is likely this finding will play a part in the future development of drugs aimed at impeding the transmission of malaria, the most widespread and destructive parasitic disease in the world.

EXPERIMENTAL PROCEDURES

Preparation of Extracts—Procedures for extracting and chromatographically purifying GAF from Anopheles stephensi mosquito tissues have been described (3). In this case GAF was prepared from 75,000 mixed male and female heads of A. stephensi (batch NFS0); storage of the final extract was in water at ~20 °C.

Analytical Procedures—High resolution electrospray interface mass spectrometry of GAF was performed at the Mass Spectrometry Laboratory of the National Center for Environmental Health at the Centers for Disease Control and Prevention (Atlanta, GA). Positive ion spectra were obtained from a Micromass 70–4F double focusing magnetic sector tandem mass spectrometer fitted with an electrospray interface. Solvent delivery was performed with HP1090 HPLC equipped with a HP diode array detector. A Vydac C_{18} column (1 × 150 mm, 90 Å, 5 μm) was used at a flow rate of 40 μl min^{-1}. HPLC solvents used were water/0.1% trifluoroacetic acid (Buffer A) and acetonitrile/0.1% trifluoroacetic acid (Buffer B). Gradient elution was obtained by a hold at 1% Buffer B for 1 min and then a linear ramp from 1 to 13% Buffer B in 19 min followed by a linear ramp to 30% Buffer B in 5 min (for eluting leucine enkephalin time marker). The effluent was monitored at 205 nm. Wavelength scans (200–600 nm) were taken for all peaks eluting from the column. Initially, low resolution scans (1000 resolution at 10% valley) were performed over the m/z range of 100–600 atomic mass units. The high resolution scans were performed over a narrow mass range to bracket the major ion (m/z = 206) with two mass reference ions from PEG-200. The mass calibrant was injected post-column just prior to and just after elution of the 206 ion to negate the effect of ion drift on mass measurement. The analyses were done in triplicate. Potential elemental compositions were deduced employing the standard Micromass OPUS software, and candidate compounds were suggested from a search of National Institute of Standards and Technology software (1994-1995 edition) supplied with the OPUS system. UV spectra and retention time of XA were determined under identical conditions of reverse phase high performance chromatography as mosquito-derived GAF. The system employed consisted of a Vydac narrow bore column (2.1 mm × 25 mm, 5 μm, 300 Å) attached to Beckman 126 pumps and a model 168 Diode Array Detector controlled by Beckman System Gold software (version 8.1). The compounds were eluted isocratically with a solvent of 5% CH_{3}CN/94.9% H_{2}O/0.1% trifluoroacetic acid at a flow rate of 0.3 ml min^{-1} and ambient temperature.

XA (Sigma) concentrations were determined from the molar extinction coefficient (ε) at 247 nm. The ε_{247} was found by dissolving 0.54 μmol (0.11 mg) of XA in 1 ml (0.54 mM) of PBS (pH 7.4) and measuring the absorbance of the solution in a Beckman DU-65 spectrophotometer. The ε_{247} = 20,988 cm^{-1} μM^{-1}. Concentrated solutions of XA were prepared in water and then adjusted to pH 7.4 with 0.5 N NaOH before addition of PBS. GAF concentrations were estimated from a standard curve of XA peak areas measured by RP-HPLC. Kynurenic acid (Sigma) stock solution was prepared by dissolving 0.12 mg (0.58 μmol) of the monohydrate form in 1 ml (0.58 mM) of PBS. Kynurenine (Sigma, 0.5 H_{2}O mol/mol) 0.32 mg (1.5 μmol) was dissolved in 1 ml of PBS (1.5 mM). Kainic acid monohydrate (Sigma) 0.47 mg (2 μmol) was dissolved in 1 ml of PBS (2 mM).

Bioassays—In vitro activity was determined using P. gallinaceum, a bird malaria parasite, and cultured P. falciparum, a human parasite, as described previously (3). Briefly, 5 μl of test substances dissolved in PBS were added to preparations of gametocytes stored in PBS, which retards gametogenesis; induction is scored by microscopically counting the number of male gametes exflagellating 15–30 min later.

RESULTS

We had previously determined that GAF has a major, singly charged ion species at m/z 206. Three runs of high resolution
voltage scans were done of the bioactive fraction over a narrow range bracketing 206 (Table I). Results were highly consistent, and a median weight of 206.0450 was chosen to do an elemental composition search, with limits on the accuracy of the mass measurement set at 20 ppm. Only compounds containing carbon, hydrogen, oxygen, nitrogen, phosphorus, or sulfur were searched; several elements with distinctive isotope distributions not evident in spectra (e.g., chlorine, bromine, and copper) were excluded from consideration. The most likely composition, at an accuracy of 0.6 ppm, was C-10, H-8, N-1, O-4; assuming that the observed ion was [M+H]+, the most likely composition of the parent species was C-10, H-7, N-1, O-4. A search of the National Institute of Standards and Technology electron ionization libraries done for all entries with nominal weight of 205 yielded 292 candidate compounds; only those with the formula C-10, H-7, N-1, O-4 were chosen for further consideration. All but four candidates could be eliminated on the basis of having weights outside the 20 ppm limit, having rare atoms unlikely to be present (e.g., silicon or boron), or having atoms known to be absent (e.g., chlorine or bromine). The four compounds included 2-isobenzazol and three isomers of quinolinecarboxylic acid, one of which, XA, commonly occurs in insects (4).

### TABLE I

**High resolution mass measurements on GAF**

GAF purified from *A. stephensi* heads was analyzed by accurate mass high resolution electrospray interface mass spectrometry. Three runs were performed employing narrow range voltage scans using PEG-200 for mass calibration (details are under “Experimental Procedures”). Measured weights are averages of two readings. Resolutions are for 10% valley.

| Run number | Measured weight | Resolution |
|------------|-----------------|------------|
| 1          | 206.0451        | 5000       |
| 2          | 206.0450        | 3700       |
| 3          | 206.0450        | 3700       |

**TABLE II**

The ability of XA and KYNA to induce gametogenesis of the malaria parasite *P. gallinaceum* compared with that of the natural signal, GAF

Induction was measured by counting at 200× magnification the number of male parasites exflagellating in a 5-μl suspension of washed, infected chicken erythrocytes 15–30 min after addition of test compounds in PBS. Data are means of triplicate matched tests; the negative control, PBS, was always 0. Statistical significance was calculated between compounds at the same concentrations; each marked value is significantly different from value immediately below. XA activated the human parasite *P. falciparum* at 325 nM (data not shown). ND, not done.

| Compound | Test concentrations |
|----------|---------------------|
|          | 1 μM | 100 μM | 10 μM | 1 μM | 500 nM | 250 nM | 125 nM | 62 nM |
| GAF      | ND   | 190    | ND    | 261  | 57    | 9     | 0     |
| XA       | 211  | 238    | 217   | 249  | 100   | 22    | 12    | 0     |
| KYNA     | 81   | 63     | 0     | 0    | ND    | ND    | ND    | ND    |

* p ≤ 0.01.

* *p* ≤ 0.05.

**Fig. 1. Analytical comparison of GAF and XA.** A. Chromatographic profiles of GAF (0.112 nmol) isolated from mosquito heads; XA (0.116 nmol); and a mixture of GAF (0.112 nmol) and XA (0.116 nmol). The method for RP-HPLC through a narrow bore C18 column is detailed under “Experimental Procedures.” B, normalized UV absorbance profiles of GAF (solid line) from mosquito heads and XA (dashed line) from RP-HPLC runs. Note that the nearly complete superposition of the two sets of data give the appearance of a single line. Inset, chemical structure of XA.
We found the chromatographic (Fig. 1A) and UV (Fig. 1B) characteristics of XA, or of a mixture of XA and GAF, to be identical to those of GAF. The ability of XA to stimulate gametogenesis in *P. gallinaceum* was comparable with that of GAF, with a threshold dose as low as 0.5 μM (Table II). XA and GAF at 1 mM also activated the human parasite *P. falciparum* at the same rates (73 and 51, respectively); XA concentrations as low as 325 nM induced prolific gametogenesis. A close analog of XA, kynurenic acid, activated gametogenesis of *P. gallinaceum* but at a maximum effect about one-third that of XA and with no induction below 100 μM (Table II). Two other products of tryptophan catabolism, kainic acid and kynurenine, had no bioactivity.

**DISCUSSION**

Gametocytes rarely constitute more than 5% of all infected red blood cells, and unlike the asexual forms, whose rapid cell division and growth force disintegration of the host erythrocyte membrane (7), the gametocytes depend on an external signal to activate escape. The survival benefit to the parasite of this requirement is clear: only mating in the gut will ensure transmission to a new host. A reduction in temperature from 37 °C to less than 30 °C is a precondition for activation but does not alone induce gametogenesis (1). We have recently shown that a single molecular species, GAF, present in both the gut and the head of a vector mosquito acts as a potent inducer of gametogenesis in a simple, protein-free, saline solution that otherwise would indefinitely prohibit activation (3). In this report we present compelling analytical evidence that XA is GAF. The bioactivity of XA is confirmatory.

XA is widely distributed in insects, where it is a byproduct of the 2,3-dioxygenase pathway of tryptophan degradation, which leads through kynurenine to, among other compounds, the ommochromes, common pigments of invertebrate eyes and integument (4). In vertebrates, XA has been found in urine as an indication of vitamin B₆ levels (5) and has been linked indirectly to some pathological conditions, such as cataract formation (6). It has not, however, been reported before to have inductive ability. How it induces gametogenesis is not yet known. Before gametogenesis begins, the parasite is separated from the erythrocyte cytoplasm by the host cell-derived parasitophorous vacuole membrane and from the external environment by the erythrocyte plasma membrane (7). There is evidence that gametogenesis is preceded by activation of the phosphoinositol cascade (8) and release of intracellular bound Ca²⁺ (9). XA, which is relatively hydrophilic, may act as a ligand at the outer, erythrocyte membrane. Notably, KYNA, which differs structurally from XA only in the absence of a hydroxyl group from position 8, also stimulated gametogenesis, although at a substantially lower level. KYNA is now known to be an endogenous, nonspecific antagonist at the excitatory amino acid receptors in vertebrate central nervous systems, as well as having a high affinity for the glycine site in the N-methyl-D-aspartate receptor complex (10); XA has been used as an inactive control in studies of KYNA kinetics.

Presumably the mosquito head has proved to be a relatively rich source of GAF because of the metabolism of eye pigment, but it is not yet clear why XA is also present in its gut. Nor is the possible contribution of XA in vertebrate serum known; few data on serum levels have been published. Nevertheless, we now have a tool for investigating the precise sequence of gametogenesis induction. Considerable effort is now focused on developing vaccines targeted at the sexual stages in the gut, which would block malaria transmission (11), and it seems likely that understanding the mechanics of induction will make possible a parallel effort to develop transmission blocking drugs.

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**REFERENCES**

1. Sinden, R. E., Butcher, G. A., Billker, O., and Fleck, S. L. (1996) *Adv. Parasitol.* 38, 53–117
2. Nijhout, M. M. (1979) *Exp. Parasitol.* 48, 75–80
3. Garcia, G. E., Wirtz, R. A., and Rosenberg, R. (1997) *Mol. Biochem. Parasitol.* 88, 127–135
4. Kayser, H. (1985) in *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Kerkut, G. A., and Gilbert, L. I., eds) Vol. 10, pp. 367–415, Pergamon Press, Oxford
5. Liu, M., Wang, G. R., Liu, T. Z., and Tsai, K. J. (1996) *Clin. Chem.* 42, 591–599
6. Aikawa, M., Carter, R., Ito, Y., and Nijhout, M. M. (1984) *J. Protozool.* 31, 403–413
7. Ogwan’g, R., Mwangi, J., Gachihi, G, Nwachukwu, A., Roberts, C. R., and Martin, S. K (1989) *Biochem. Pharmacol.* 48, 1601–1606
8. Kawamoto, F., Fujikura, H., Murakami, R. I., Syafruddin, Hagiwara, M., Ishikawa, T., and Hidaka, H. (1993) *Eur. J. Cell Biol.* 60, 101–107
9. Kesler, M., Terramani, T., Lynch, G., and Baudry, M. (1989) *J. Neurochem.* 52, 1319–1328
10. Williamson, K. C., Fujikura, H., Aikawa, M., and Kaslow, D. C. (1996) *Mol. Biochem. Parasitol.* 78, 161–169