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Fractions of an antimalarial neem-leaf extract have activities superior to chloroquine, and are gametocytocidal

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The antimalarial activities of two fractions (IRDN-A and IRDN-B) of an extract from the leaves of the neem tree (Azadirachta indica) were compared with those of chloroquine, in in-vitro assays against Plasmodium falciparum. The sexual stages of a chloroquine-sensitive clone (ITG2F6) and a chloroquine-resistant isolate (W2) and the gametocytes of the NF 54 (BD-7) isolates of P. falciparum were used as the drug targets. Activity against the sexual stages was generally evaluated as the concentrations inhibiting the parasitaemias recorded in the control cultures, after an incubation of 48-72 h, by 50% (IC50) or 100% (IC100). For the ITG2F6 strain, the IC50 and IC100 (in pgl/ml) were, respectively, and for IRDN-A, and for IRDN-B, and 1.0 for chloroquine. The corresponding values for the W2 strain were 10.0 and 1.0 for IRDN-A, and 10.0 and >100 for chloroquine (even at 100 pgl/ml, chloroquine only inhibited the parasitaemia by 85%).

Each of the two neem-leaf fractions lysed 50% and 100% of developing gametocytes, at 10-4 and 10-1 pgl/ml, respectively; and 50% and 100% of mature gametocytes at 10-3 and 10-2 pgl/ml, respectively. If they are found safe and effective in vivo, the neem-leaf fractions may form the basis of new antimalarial drugs that not only cure chloroquine-sensitive and chloroquine-resistant malaria but also markedly reduce transmission.

The significant advances made, in the last two decades, in the treatment of human malaria have been achieved partly by the development of new antimalarial drugs, and partly by the use of combinations of the older drugs (Luxemburger et al., 1995; Looareesuwan et al., 1999; Mutabingwa et al., 2001; WHO, 2001). Unfortunately, the development of drug resistance by the parasites that cause the disease has meant that malarial morbidity and mortality remain high, with unrelenting, gametocyte-mediated transmission in many endemic areas (Anon., 1978; Bjorkman and Phillips-Howard, 1990). In the continuing search for compounds that might be used as new treatments for malaria, several plants that have been reported to have antimalarial activity have been investigated. The leaves of the neem tree (Azadirachta indica), for example, have been found to contain compounds with antimalarial activity (Karanja, 1997; Badam et al., 1997). In the present study, the antimalarial activities of two fractions of an acetone/water extract of the neem tree (Azadirachta indica) were compared with those of chloroquine, in in-vitro assays against Plasmodium falciparum. The sexual stages of a chloroquine-sensitive clone (ITG2F6) and a chloroquine-resistant isolate (W2) and the gametocytes of the NF 54 (BD-7) isolates of P. falciparum were used as the drug targets. Activity against the sexual stages was generally evaluated as the concentrations inhibiting the parasitaemias recorded in the control cultures, after an incubation of 48-72 h, by 50% (IC50) or 100% (IC100). For the ITG2F6 strain, the IC50 and IC100 (in pgl/ml) were, respectively, and for IRDN-A, and for IRDN-B, and 1.0 for chloroquine. The corresponding values for the W2 strain were 10.0 and 1.0 for IRDN-A, and 10.0 and >100 for chloroquine (even at 100 pgl/ml, chloroquine only inhibited the parasitaemia by 85%).

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neem leaves were compared with those of chloroquine (CQ), in in-vitro assays involving the asexual forms of a CQ-sensitive strain and a CQ-resistant strain of Plasmodium falciparum. The effects of the fractions on cultures of developing and mature gametocytes were also explored.

MATERIALS AND METHODS

Extraction and Fractionation

Neem leaves collected in Nigeria were extracted in a mixture of acetone and water (1:1, by vol.), as previously described (Udeinya, 1993). The bottom and top layers of the crude extract were separated before the solvents were evaporated off and the residues allowed to cool. The residue from the bottom layer of the crude extract (IRAB), which, in preliminary trials, appeared to have greater antimalarial activity in vitro than the residue from the top layer (unpubl. obs.), was then fractionated by standard, high-performance liquid chromatography (HPLC). A prepacked,μBondapak™ C18, reverse-phase column (Millipore Corporation, Milford, MA) and, as the mobile phase, a mixture of acetonitrile and methanol were used for the chromatography. The eluted fractions were scanned with an ultra-violet detector coupled to an online computing integrator. The two fractions with the largest ‘peak areas’, named IRDN-A and IRDN-B, were collected, dried in a vacuum evaporator (Savant Instruments, Farmingdale, NY), and stored in glass vials at 0-8°C.

Parasite Cultures

ASEXUAL FORMS

Asexual forms of the CQ-sensitive ITG2F6 clone of P. falciparum and of the CQ-resistant WD isolate were cultured continuously (Trager and Jensen, 1976). Tests were carried out in 24-well culture plates (Costa, Cambridge, MA), with cultures started at 0.5% parasitaemia and 2% haematocrit.

SEXUAL FORMS

Gametocytes of the NF 54 (BD-7) clone of P. falciparum (Burkot et al., 1984) were cultured and induced as previously described (Fediba and Vanderberg, 1982). Routine cultures were maintained at 0.1%-1% gametocytaemia and 3% haematocrit. Each of these routine cultures usually contained gametocytes at various stages of development. For the assays of gametocytocidal activity, synchronous cultures were obtained by sorbitol treatment (Lambros and Vanderberg, 1979) and maintained in 24-well culture plates. Tests were carried out using cultures containing immature, developing gametocytes (at 5% gametocytaemia) or only mature (stage 5 or higher) gametocytes (at 8% gametocytaemia).

Assay Procedures

For the tests with the asexual parasites, IRDN-A, IRDN-B and CQ were each dissolved in dimethyl sulfoxide (DMSO), diluted with culture medium, and added to the cultures to give final concentrations of 10⁻⁵, 10⁻⁴, 1.0 and 100 μg/ml. For the tests with the gametocytes, IRDN-A and IRDN-B were added to the cultures to give final concentrations of 10⁻⁵, 10⁻⁴, 1.0, 10, 100 and 1000 μg/ml. All drug concentrations were tested in triplicate in each set of plates and each experiment was conducted four times. As controls for each test, CQ and fraction-free DMSO was added to some cultures to give concentrations equivalent to those in the wells that contained one of the test compounds. Only CQ and the fraction found most active against ITG2F6 were tested against the CQ-resistant W2 parasites. All cultures were incubated at 37°C for 48 h (W2 and NF 54) or 72 h (ITG2F6). The medium in each culture was replaced every 24 h, when bloodsmears for the microscopic evaluation of parasitaemia or gametocytaemia in each culture were also prepared.
Concentration (pg/ml)

**FIG. 1.** Inhibition of the cultured uophozoites and schizonts of the chloroquine-sensitive ITG2F6 clone of Plasmodium falciparum, by various concentrations of chloroquine (•) or the IRDN-A (□) or IRDN-B (○) fractions of a neem-leaf extract. Parasitaemias in the test and control cultures were compared after incubation for 72 h.

**Evaluating Antimalarial Activities**

At the end of the incubation, the parasitaemias or gametocytaemias (PNG) recorded in each treated culture (i.e. a culture containing a test compound) was compared with that in the corresponding control culture. The two values compared were used to evaluate the percentage inhibition (asexual forms) or percentage lysis (gametocytes) caused by the compound at the concentration used, as $\frac{100(P_G_{\text{Control}} - P_G_{\text{Treated}})}{P_G_{\text{Control}}}$. The concentration of a compound causing approximately 50% inhibition/lysis and the lowest tested concentration causing 100% inhibition/lysis were considered to be the approximate $IC_{50}$ and $IC_{100}$ for that compound, respectively.

**RESULTS**

**Controls**

In the absence of CQ or a neem-leaf fraction, the asexual parasitaemias of ITG2F6 rose from 0.5% to 9 ± 0.7% between the initiation of the culture and the end of incubation, whereas those of W2 rose from 0.5% to 6 ± 0.7%. In the control cultures of developing gametocytes, gametocytaemias rose only from 5% to 6% during incubation, and the gametocytaemias in the cultures of mature gametocytes remained unchanged, at 8%. All the gametocytes in the control cultures appeared normal and well developed, with both micro- and macro-gametocytes present in the mature cultures.

**Treated Cultures**

**ASEXUAL FORMS**

IRDN-A, IRDN-B and CQ inhibited the asexual parasitaemias of the CQ-sensitive ITG2F6 to varying degrees, with $IC_{100}$ of $10^{-3}$, $10^{-2}$ and 1.0 μg/ml, respectively (Fig. 1). The effects of the drugs on parasitaemia were dose-dependent. At $10^{-6}$ μg/ml, for example, IRDN-A caused only 15% inhibition, whereas no asexual forms were seen, at the end of the incubation, in the cultures with 10 μg IRDN-A/ml. At $10^{-4}$ and $10^{-3}$ μg/ml, IRDN-B caused 35% and 100% inhibition, respectively, while CQ at $10^{-2}$ and 1.0 μg/ml caused 55% and 100% inhibition, respectively. The approximate $IC_{50}$ for IRDN-A, IRDN-B and CQ tested against ITG2F6 were $10^{-3}$, $10^{-4}$ and $10^{-3}$ μg/ml, respectively.

Against the asexual forms of CQ-resistant W2 isolate, IRDN-A at $10^{-4}$, $10^{-4}$, $10^{-2}$ and 1.0 μg/ml caused 32%, 80%, 89% and 100% inhibition, respectively (Fig. 2), whereas CQ at $10^{-4}$, $10^{-4}$, $10^{-3}$, 1.0 and 100 μg/ml inhibited by 0%, 0%, 15%, 20%,
and 87%, respectively. The approximate IC\textsubscript{50} against W2 were $10^{-3}$ µg/ml for IRDN-A and 10.0 µg/ml for CQ. The corresponding IC\textsubscript{100} were 1.0 µg/ml for IRDN-A and >100 µg/ml for CQ (even at 100 µg/ml, the highest concentration tested, CQ only caused 85% inhibition).

The IC\textsubscript{50} and IC\textsubscript{100} of IRDN-A and IRDN-B were statistically significant (P<0.01 for each). With the W2 cultures, the inhibitions caused by IRDN-A and IRDN-B were statistically significant (P<0.01 for each).

### Sexual Forms (Gametocytes)

Both neem-leaf fractions caused the lysis of developing and mature gametocytes in vitro, leaving gametocyte fragments and loose malarial pigment in the cultures after incubation for 48 h. Most of the few gametocytes that survived in the treated cultures appeared abnormal and there was no evidence of gametocyte maturation during the incubation. The reductions in gametocytaemia observed in the cultures of developing and mature gametocytes exposed to IRDN-A and IRDN-B were all statistically significant (P<0.01 for each).

### DISCUSSION

In the assays with the asexual-stage cultures of CQ-sensitive ITG2F6, IRDN-B and particularly IRDN-A exhibited good antimalarial activity. The approximate IC\textsubscript{50} (10\textsuperscript{-5} µg/ml) and IC\textsubscript{100} (10\textsuperscript{-4} µg/ml) for
IRDN-A were remarkably low, and much lower (P<0.01 for each) than the corresponding values for CQ, which were, respectively, 1000- and 10,000-fold higher. There is, presumably, some difference between IRDN-A and the slightly less active IRDN-B in the concentration or nature of the active ingredient in each fraction and/or its mode of action.

Encouragingly, in terms of its IC50, IRDN-A was as active against the asexual stages of the CQ-resistant W2 isolate as against those of the CQ-sensitive clone. Even more encouragingly, IRDN-A and IRDN-B possessed similar and significant activities against both immature and mature gametocytes of *P. falciparum* (with a consistent IC50 of about 10^{-5} μg/ml). The between-fraction similarities of the dose-response curves for the assays with immature (Fig. 3) or mature gametocytes (Fig. 4) are marked, and may be an indication that the gametocytocidal compound in IRDN-A has a similar mode of action to that in IRDN-B (and that the gametocytocidal activities of the two fractions are virtually identical). Given the survival of the uninfected erythrocytes in all the gametocyte cultures, the presence of large amounts of gametocyte fragments and malarial pigment in the treated cultures is evidence that the gametocytocidal compounds in the neem-leaf fractions probably cause gametocyte-specific lysis.

In conclusion, in vitro, both IRDN-A and IRDN-B possess strong antimalarial activity that is significantly superior to that of CQ and, for IRDN-A at least, equally active against the sexual stages of CQ-sensitive and CQ-resistant *P. falciparum*. Furthermore, both fractions have significant anti-gametocyte activity in vitro, causing the lysis of developing and mature gametocytes alike, without affecting uninfected erythrocytes. If IRDN-A, IRDN-B or similar neem-derived fractions are found to be as effective in vivo, without causing any adverse effects, they may well form the basis of new drugs for the effective treatment of *P. falciparum* malaria (that may concomitantly help to reduce the gametocyte-dependent mosquito transmission of malaria).

REFERENCES
