INTRODUCTION

Dengue fever is one of the most important arthropod-borne viruses transmitted by Aedes aegypti in tropical countries. Dengue virus belongs to the Flaviviridae family and includes four antigenic serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) which are responsible for the disease. The clinical patterns range from mild febrile illness (fever, muscular pain, headaches and digestive disorders) to severe forms of infection (haemorrhage, shock syndrome, encephalitis) which may lead to death. In the absence of vaccines and specific treatments, prevention is primarily recommended by integrated vector control strategies (WHO, 1997).

In order to search for new antiviral drugs, bioactive metabolites isolated from marine invertebrates living in New-Caledonian waters were investigated. Antiviral properties of gymnochromes against dengue viruses were previously reported (Laille et al., 1998). Gymnochromes are brominated compounds with an hypericin core obtained from a fossil crinoïd Gymnochrinus richeri (De Riccardis et al., 1991). Hypericin and pseudohypericin are known for their high potency against retroviruses (Meruelo et al., 1988). The virucidal and antiviral photoactivities of hypericin were first demonstrated on inactivated murine cytomegalovirus, Sindbis virus and human immunodeficiency virus type 1 (Hudson et al., 1991). Photosensitization of hypericin is also required for the inactivation of Equine Infectious Anemia Virus (Carpenter and Kraus, 1991). Furthermore, Hudson et al (1999) demonstrated the effect of light exposure on the antiviral activities of hypericin and its brominated derivatives against both Herpes simplex and Influenza viruses. These compounds have extended-electron systems which are responsible for singlet oxygen production upon photoexcitation with visible light. Similarly (Sauviat et al., 2001) showed that photoexcitation of gymnochrome A blocked the background K+ current in frog atrial heart muscle and altered transmembrane currents. The aim of this study was to compare the viru-
cidal and antiviral photoactivities of hypericin, tetrabromohypericin and gymnochrome B on dengue virus.

MATERIALS AND METHODS

Chemicals
Chemicals were obtained from Sigma-Aldrich (analytical grade). Hypericin was purchased from Extrasynthese (Genay, France). Tetrabromohypericin was obtained from hypericin as follows (Itaya et al, 1999): a solution of Br₂ in acetic acid (three times 15 µl, 90 µmoles) was added to a stirred solution of hypericin (19.8 µmoles) and sodium acetate AcONa (62 µmoles) in 1 ml acetic acid at room temperature, protected from light. The reaction was monitored by electrospray ionization mass spectra (ESI-MS) until the bromination was completed to the tetrabromine derivative after 75 min, using an esquire-liquid chromatography apparatus in negative mode: [M-H], 79Br, hypericin: 503.2 mu, Br-hypericin: 581.0 mu, Br₂-hypericin: 659.0 mu, Br₃-hypericin: 736.8 mu, Br₄-hypericin: 814.8 mu. The resulting purple red solution was diluted in 40 ml of chloroform and the solution was washed with water. The chloroform solution was dried over anhydrous sodium sulfate and the solvent vacuum-evaporated. The 2,5,9,12-tetrabromohypericin was obtained as a dark solid (7 mg, 43% overall yield) and controlled by thin layer chromatography and ESI-MS in negative mode. UV-Vis spectrum using a Beckman DU-600 spectrophotometer (λmax in MeOH: 291, 327, 391, 483, 551, 594) was consistent with Falk’s data (Falk and Schmitzberger, 1993). Gymnochrome B was isolated from the deep water stalked fossil crinoïd Gymnochrinus richeri collected on the seamounts of the Norfolk Ridge and purified as previously described (De Riccardis et al, 1991). The compounds were dissolved in dimethyl sulfoxide (DMSO), and diluted in culture medium to obtain stock solutions at 1 mg/ml and up to a 1% maximum final DMSO concentration and stored at 4°C in the dark. The tested doses ranged from 50 to 0.001 µg/ml.

Virus stocks
DEN-4 (H-241 prototype strain) and DEN-2 (New Guinea C prototype strain) virus stocks were prepared using infected brains of suckling mice, and titrated using serial dilutions of virus stock by plaque assays using Porcine PS cell layers. Titration plaques were counted 5 days post-inoculation, preceded by gentian violet coloration. The concentration of the viral suspensions was expressed as the number of Plaque Forming Units (PFU) per mL. Adequate aliquots of virus suspensions were stored at -80°C. DEN-1 (strain 16007), DEN-2 (strain 16681), DEN-3 (strain 16562), DEN-4 (strain D4/1036) and Japanese Encephalitis Virus (Beijing strain) were also prepared and titrated using LLCMK2 cells before and after tests with the compounds in Thailand (Russel et al, 1967).

Virucidal and antiviral assays
A previously described method with slight modifications was used to evaluate the virucidal and antiviral activities of the products by plaque reduction assays using PS cells in 24-well plates for all virus strains tested. Briefly the compounds were tested using ten-fold dilutions in the presence of 50-100 PFU. For the virucidal assays, the virus-tested compound mixture contained in a test tube was incubated for 30 minutes at 37°C prior to inoculation. The gelled 3% carboxymethylcellulose culture medium was poured 1 h post-inoculation. In antiviral assays, the tested compound was diluted in the carboxymethylcellulose medium and applied 1 hour post-inoculation with the virus. Plaques were counted 5 days post-inoculation by staining the cells with gentian violet and the percentage of plaque reduction calculated versus the 0% reduction control. For the virucidal experiments conducted with light, an irradiation period of 30 min with a 60 watt bulb (6,750 lux) placed at a distance of 30 cm, was applied to the virus-gymnochrome mixture prior to inoculation. For the antiviral experiments conducted in the absence of light, the plates were directly incubated after depositing the tested compounds. In the presence of light, the plates were irradiated for 30 minutes before incubation.

Statistical analysis
For each concentration, the mean value of the percentage of inhibition was calculated. Curves of the percentage of inhibition versus the log of the concentration of the tested products...
for both assays (virucidal and antiviral) with or without light were then plotted. In the case of linearity according to the F-test, the dose of product able to reduce 50% of the number of foci was calculated by interpolation. Such a dose was expressed as the 50% Effective Dose (ED$_{50}$). For either virucidal or antiviral effects, the comparison of the overall level of potency between the three products tested at different concentrations and in the absence light was carried out by the analysis of covariance (ANCOVA). The difference between the three products was assessed upon the F value with a risk <0.05. A similar comparison was performed for assays conducted in the presence of light. Furthermore, the potency of each product for either virucidal or antiviral effects “without light” versus “with light” was compared using the ANCOVA analysis according to the F value with a risk < 0.05.

Comparison of either virucidal or antiviral effects of the three products in the absence of light

The results expressed as their ED$_{50}$ values are summarized in Table 1. For either virucidal or antiviral DEN-4 assays without light, the three products demonstrated significant differences in their potency levels (p=0.001). These activities were log dose-dependent upon a linear regression curve (Fig 2a and 2b). The decreasing potency of the virucidal effects was in the following order: gymnochrome B (ED$_{50}$ = 0.21 nM/ml), hypericin (ED$_{50}$ = 6.2 nM/ml) and tetrabromohypericin (ED$_{50}$ = 14 nM/ml). For antiviral effects, the order was the same: gymnochrome B (ED$_{50}$ = 0.56 nM/ml), hypericin (20 < ED$_{50}$ < 99.4 nM/ml) and tetrabromohypericin (ED$_{50}$ > 61 nM/ml).

### Results

Chemical structure of the tested compounds

The structures of hypericin, tetrabromohypericin, gymnochrome A and gymnochrome B are depicted in Fig 1. Gymnochrome A, whose molecule possesses four bromine atoms, is closer to tetrabromohypericin than gymnochrome B, which possesses three bromine atoms. Due to its limited availability, gymnochrome A could not be used for this study. However, preliminary tests did not show significant differences between their antiviral activities.

#### Table 1

<table>
<thead>
<tr>
<th>Product</th>
<th>Virucidal With light</th>
<th>Antiviral With light</th>
<th>Virucidal Without light</th>
<th>Antiviral Without light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypericin</td>
<td>1.8 (13)</td>
<td>0.6 (8)</td>
<td>6.2 (12)</td>
<td>20&lt;ED$_{50}$&lt;99.4 (12)</td>
</tr>
<tr>
<td>Tetrabromohypericin</td>
<td>2.8 (10)</td>
<td>3.7 (13)</td>
<td>14 (10)</td>
<td>&gt; 61 (13)</td>
</tr>
<tr>
<td>Gymnochrome B</td>
<td>0.042 (30)</td>
<td>0.029 (24)</td>
<td>0.21 (29)</td>
<td>0.56 (29)</td>
</tr>
</tbody>
</table>

ED$_{50}$ = 50% Effective doses; () = number of duplicate assays at concentrations ranging between 0.001 and 50 µg/ml
Comparison of either the virucidal or the antivi-
rnal effects of the three products in the presence
of light

For either virucidal or antiviral DEN-4 as-
says with light, the three products showed sig-
nificantly different potency levels (p=0.001) (Table
1). These activities were log dose-dependent
upon a linear regression curve (Fig 2a and 2b).
The virucidal potency are listed in decreasing
order as follows: gymnochrome B (ED$_{50}$ = 0.042
nM/ml), hypericin (ED$_{50}$ = 1.8 nM/ml) and
tetrabromohypericin (ED$_{50}$ = 2.8 nM/ml). The
same pattern of relative potency was observed
in the antiviral assays: gymnochrome B (ED$_{50}$ =
0.029 nM/ml), hypericin (ED$_{50}$ = 0.6 nM/ml) and
tetrabromohypericin (ED$_{50}$ = 3.7 nM/ml).

Influence of light

Light increased the virucidal potency of
each of the three products by a factor of 3.5,
4.9 and 5.2, for hypericin, tetrabromohypericin
and gymnochrome B, respectively (ED$_{50}$ values).
The antiviral effects were much higher in the
presence of light for gymnochrome B, whose
ED$_{50}$ decreased by 19.3 times.

Mechanism of action

Hypericin derivatives showed virucidal and
antiviral activities against DEN-4 prototype strain
(H-241). In addition, similar effects were ob-
served with the DEN-2 prototype strain (New
Guinea C), Thai Dengue virus strains (see virus
stocks) and another flavivirus, the Japanese
Encephalitis Virus (data not shown). The virucidal
activity of gymnochrome was not observed in
experiments previously carried out in our labo-
atory (Laille et al, 1998), because the effect of
light was not investigated.

DISCUSSION

As reported by Hudson et al (1999), for Her-
pes simplex and Influenza viruses, the overall
activity of the three compounds (hypericin,
tetrabromohypericin and gymnochrome B)
against Dengue virus was potentialized upon
exposure to light, especially for gymnochrome
B in both virucidal and antiviral assays. As for
Herpes simplex and Influenza viruses, the least
active product against the Dengue virus was
tetrabromohypericin. Gymnochrome B was the
most active product against the Dengue virus,
while it had the same potency as hypericin and
dibromohypericin against Herpes simplex vi-
ruses. These observations point out the role of
the gymnochrome side chains and their activi-
ties against the Dengue virus, whereas the Her-
pes simplex and Influenza viruses are probably
not sensitive to products with this chemical
structure. Possible differences in the mechanism
of action of these products against viruses have
to be considered.

Studies of the structure-activity relationship
of hypericin and related analogues have been
described by Cohen et al (1996). Antiviral activ-
ity against Herpes simplex viruses was negatively
correlated with the level of substitution of cho-
lorine in the hypericin structure in position 7 (7,7'-
dichlorohypericin). Studies of the antiretroviral
activities of quinones, hypericin and pseudohy-
pericin against the equine infectious anemia vi-
rus (a retrovirus which has been used as a model
for HIV), showed that the complete ring struc-
ture of hypericin is required for photoactivation-
dependant antiviral activity (Kraus et al, 1990).
For some synthetic 1,4-phenanthrenequinones,
the antiretroviral activity occurs through a
mechanism independent of photoactivation
(Kraus et al, 2000; Fehr et al, 1994) suggesting
the possibility of a light-independent mechanism.
They have shown that the oxygen singlet does
not play a major role in the antiviral activity of
hypericin.

In our experiments, the differences between
the activities of hypericin and gymnochrome B
were not due to the photophysical aspects. The
fact that they have the same absorption spectra
with a strong maximum at approximately 590
nm, and that the differences between their ac-
tivities exist in the absence of light, suggests
that the side chains are responsible of an addi-
tional effect, or an enhanced photoactive effect
of the hypericin core.

This result does not depend on bromine
substitution. In order to assess whether the ob-
served activities are specific or general toxic ef-
teffects, we calculated an experimental selectivity
index (SI), defined as the ratio of cytotoxicity to
antiviral or virucidal activity. During the antiviral
experiments, in the absence of light exposure,
gymnochrome B showed cytotoxic effects on porcine cells at a concentration of 50 µg/ml (58 nM/ml); upon light exposure, the cytotoxic effects were observed at a concentration of 1 µg/mL (1.2 nM/ml). Therefore the SI was approximately 100 without light and 40 with light. For the virucidal experiments without or with light exposure, cytotoxicity was absent at 50 µg/ml, consequently the SI was higher (superior to 273 and 1404, respectively) than that for the antiviral experiments. For the antiviral experiments, light favors the cytotoxic effect to a greater extent than the antiviral activity.

In summary, gymnochrome B exhibits potent virucidal and antiviral activities due both to a photoactivity of its brominated hypericin core and to another mechanism linked to the side chains.

REFERENCES


