STRUCTURE-ACTIVITY RELATIONSHIPS OF DENGUE ANTIVIRAL POLYCYCLIC QUINONES

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Abstract. The virucidal and antiviral photoactivities of three compounds, hypericin, tetrabromohypericin and gymnochrome B, were evaluated against dengue viruses. All the three products were active, and both the virucidal and antiviral activities were enhanced by light. Gymnochrome B was more potent than hypericin and tetrabromohypericin. The presence of the side chains on the hypericin core of gymnochromes appears to be beneficial for both virucidal and antiviral activities. This enhanced activity is likely to be linked to a complementary mechanism independent of photoactivation.

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-

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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 Br_2 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], ^{79}Br, hypericin: 503.2 mu, Br-hypericin: 581.0 mu, Br_2-hypericin: 659.0 mu, Br_3-hypericin: 736.8 mu, Br_4-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 4ºC 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 -80ºC. 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 37ºC 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.

Stastistical 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.

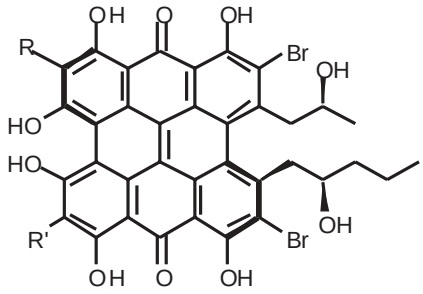
RESULTS

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.

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).



Gymnochrome A : R = R' = Br
Gymnochrome B : R = H; R' = Br or R = Br; R' = H

Fig 1–Structure of gymnochrome.

Table1
Virucidal and antiviral effects against dengue-4 viruses of the tested products with and without light.

Product	With light		Without light	
	Virucidal	Antiviral ED_{50} (nM/ml)	Virucidal	Antiviral ED_{50} (nM/ml)
Hypericin	1.8 (13)	0.6 (8)	6.2 (12)	$20<ED_{50}<99.4$ (12)
Tetrabromohypericin	2.8 (10)	3.7 (13)	14 (10)	> 61 (13)
Gymnochrome B	0.042 (30)	0.029 (24)	0.21 (29)	0.56 (29)

ED50 = 50% Effective doses; ( ) = number of duplicate assays at concentrations ranging between 0.001 and 50 μg/ml

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

For either virucidal or antiviral DEN-4 assays with light, the three products showed significantly 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 observed 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 laboratory (Laille ***et al,*** 1998). because the effect of light was not investigated.

DISCUSSION

As reported by Hudson ***et al*** (1999), for ***Herpes 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*** viruses. These observations point out the role of the gymnochrome side chains and their activities against the dengue virus, whereas the ***Herpes 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 activity against ***Herpes simplex*** viruses was negatively correlated with the level of substitution of chlorine in the hypericin structure in position 7 (7,7'-dichlorohypericin). Studies of the antiretroviral activities of quinones, hypericin and pseudohypericin against the equine infectious anemia virus (a retrovirus which has been used as a model for HIV), showed that the complete ring structure 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 activities exist in the absence of light, suggests that the side chains are responsible of an additional effect, or an enhanced photoactive effect of the hypericin core.

This result does not depend on bromine substitution. In order to assess whether the observed activities are specific or general toxic effects, 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.

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