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# Antimalarial Activity of Crambescidin 800 and Synthetic Analogues against Liver and Blood Stage of *Plasmodium* sp.

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***This article is dedicated to the memory of the late Prof. Kenneth L. Rinehart who first worked on crambescidin.***

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**Abstract** Structural features associated with the antimalarial activity of the marine natural product crambescidin 800 were studied using synthetic analogues of the related compound ptilomycalin A. The study suggests that the guanidine moiety is cytotoxic, whereas the spermidine-containing aliphatic chain increases activity. The most active analogue, compound **11**, had *in vitro* activity against *Plasmodium falciparum* strain 3D7 ($IC_{50}$=490 nM) that was stronger than the *in vitro* activity against murine L5178Y cells ($IC_{50}$=8.5∼59 $\mu$M). *In vitro* growth inhibition of liver stages of *P. yoelii yoelii* in mouse hepatocytes was observed ($IC_{50}$=9.2 $\mu$M). The compound did not significantly prolong median survival time after a single subcutaneous administration of 80 mg/kg in *P. berghei*-infected mice. Compound **11** did not cause DNA fragmentation in an *in vitro* micronucleus assay.

**Keywords** malaria, natural product, crambescidin, *Plasmodium*

**Description**

Crambescidin 800 was isolated from a *Mycophora* sp. sponge. The methanol extract was partitioned with hexane, dichloromethane (DCM) and chloroform. The DCM fraction was chromatographed successively on Sephadex LH20 (101.6×2.5 cm, 100% methanol, 2 ml/minute, elution volume ($V_e$)=280 ml) and C18 columns (gravity column, 50.8×2.5 cm, methanol/water 9 : 1, 1 ml/minute, $V_e$=33 ml) yielding a bioactive fraction (55.4 mg) whose $^{1}$H- and $^{13}$C-NMR shifts and mass spectrum data matched previously reported values for crambescidin 800 [1].

Analogues had been synthesized as described and were provided (by P. J. Murphy) as pure crystalline solids [2∼5]. The structures are shown in Fig. 1.

The structures of crambescidin 800, ptilomycalin A, and the synthetic compound **11** are shown in Fig. 2.

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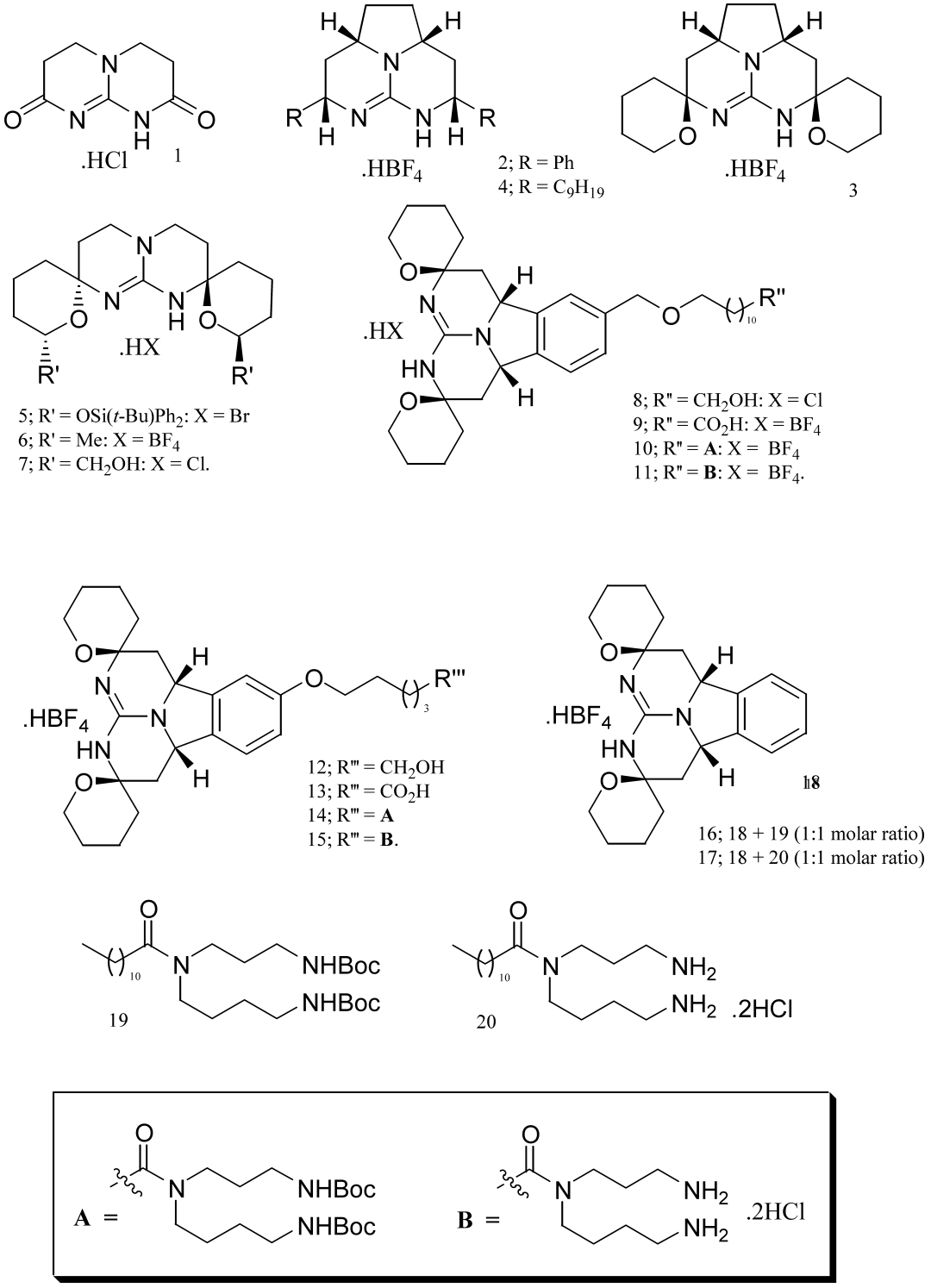


**Fig. 1** Synthetic crambescidin analogues.

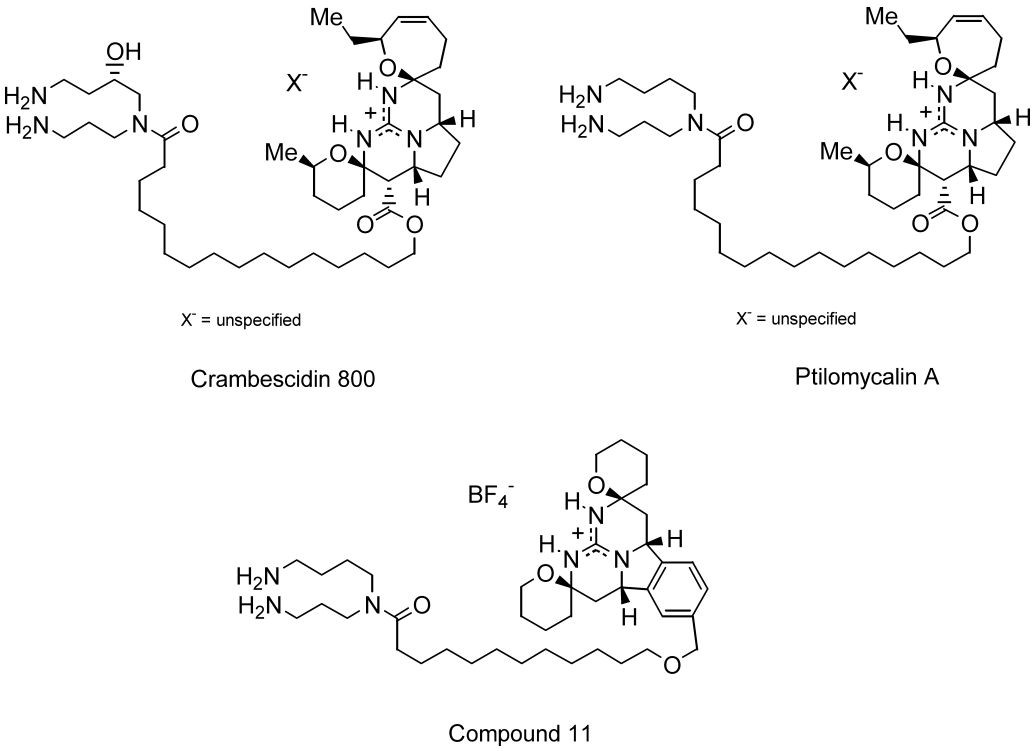


**Fig. 2** Structures of crambescidin 800, ptilomycalin A, and the analogue compound **11**.
Crambescidin LR-MS (pos-FAB): 801 ($MH^+$), $C_{45}H_{81}N_6O_6$.

**Antimalaria Assay against the Erythrocytic Stage of *Plasmodium falciparum***

*P. falciparum* strains (chloroquine-sensitive 3D7w and chloroquine-resistant FCR3) were maintained in culture as described [6]. Crambescidin 800 and synthetic analogues were tested against the parasites using the tritiated hypoxanthine assay for drug sensitivity [7]. Scintillation counts-per-minute (cpm) were adjusted relative to controls to obtain a %inhibition, p, which was transformed using the arcsine transformation, $p' = \arcsin \sqrt{p}$, as described [8]. The $IC_{50}$ was then estimated by linear regression of $p'$ versus log nM of compound. The significance of the differences between the curves defined by these data points was tested by a two-factor ANOVA using Statview® (SAS Institute, USA). The F statistic was used, with $\alpha = 0.05$.

Crambescidin 800 showed *in vitro* activity against FCR3 ($IC_{50}$=240 nM, CI 140～340 nM) and 3D7 ($IC_{50}$=160 nM, CI 100～230 nM). This activity is comparable to quinine ($IC_{50}$=290 nM, CI 200～350 nM). The *in vitro* $IC_{50}$'s of the synthetic analogues against chloroquine-sensitive strain 3D7 are given in Table 1.

Compound **11** was the most active synthetic analogue. Its *in vitro* antimalarial activity was comparable to quinine and slightly less than that of crambescidin 800.

**Antimalaria *in Vivo* Assay**

C57BL/6N mice (age 6 weeks, Charles River, France) were inoculated intraperitoneally with $1 \times 10^6$ *P. berghei* (Clone 1.49L of ANKA strain, a kind gift of Dr. D. Walliker, Institute of Genetics, Edinburgh, UK) parasites (day 0). Compound **11** and quinine were dissolved in peanut oil containing 10% DMSO, at a concentration of 20 and 10 mg/kg, respectively. A single subcutaneous dose of 80 mg/kg of compound **11** and 40 mg/kg of quinine (a high non-toxic dose according to Schneider [9]) were administered on day 3 after infection. Negative control was 10% DMSO in peanut oil. Positive control was a subcutaneous administration on day 3 with 20 mg/kg of heptyl prodigiosin in peanut oil [10]. There were eight (8) mice per group. Parasitemia was counted daily using thin smear and Giemsa staining.

Survival curves were determined with the method of Kaplan-Meier using Statview®. The Mantel-Cox log rank test was used to evaluate the chi-square and p values of the curves with $\alpha = 0.05$. Parasite counts were compared with the solvent control group using the $\chi^2$ test with $\alpha = 0.05$.

Compound **11** (80 mg/kg) and quinine (40 mg/kg) did not retard the rise in parasitemia, nor did they increase the median survival time. However, the parasitemia in mice

**Table 1** Antimalarial activity of ptilomycalin A analogues against chloroquine-sensitive of blood stage (3D7 clone) of *P. falciparum*

| Compound | Formula | Mol wt | $IC_{50}$, average and 95%CI (nM) |
|---|---|---|---|
| **1** | $C_7H_9O_2N_3 \cdot HCl$ | 203.628 | >50000 |
| **2** | $C_{21}H_{23}N_3 \cdot HBF_4$ | 405.245 | >25000 |
| **3** | $C_{17}H_{27}O_2N_3 \cdot HBF_4$ | 393.230 | >25000 |
| **4** | $C_{27}H_{51}N_3 \cdot HBF_4$ | 505.532 | 4300 (3400~5500) |
| **5** | $C_{17}H_{27}O_2N_3(OTBDPS)_2 \cdot HBr$ | 897.153 | 850 (490~1200) |
| **6** | $C_{17}H_{29}O_2N_3 \cdot HBF_4$ | 395.246 | 6200 (3300~16000) |
| **7** | $C_{17}H_{29}O_4N_3 \cdot HCl$ | 375.894 | >25000 |
| **8** | $C_{34}H_{54}O_4N_3Cl$ | 604.271 | 740 (510~1000) |
| **9** | $C_{34}H_{52}O_5N_3BF_4$ | 669.604 | >15000 |
| **10** | $C_{41}H_{67}O_4N_6(Boc)_2BF_4$ | 997.070 | 2800 (2400~3600) |
| **11** | $C_{41}H_{69}O_4N_6BF_4 \cdot 2HCl$ | 869.759 | 490 (300~730) |
| **12** | $C_{27}H_{40}O_4N_3BF_4$ | 557.433 | 3700 (3000~4700) |
| **13** | $C_{27}H_{38}O_5N_3BF_4$ | 571.416 | >18000 |
| **14** | $C_{34}H_{53}O_4N_6(Boc)_2BF_4$ | 898.882 | 5700 (5300~6100) |
| **15** | $C_{34}H_{55}O_4N_6BF_4 \cdot 2HCl$ | 771.571 | >13000 |
| **16** | $C_{21}H_{28}O_2N_3BF_4 + C_{19}H_{39}ON_3(Boc)_2$ | 969.060 | 3200 (2400~4600) |
| **17** | $C_{21}H_{28}O_2N_3BF_4 + C_{19}H_{41}ON_3 2HCl$ | 841.749 | 2100 (1400~2900) |
| **18** | $C_{21}H_{28}O_2N_3BF_4$ | 441.274 | 2300 (1800~3300) |
| **19** | $C_{19}H_{39}ON_3(Boc)_2$ | 527.785 | >19000 |
| **20** | $C_{19}H_{41}ON_3$ | 311.554 | >10000 |

Notes:

1. OTBDPS, $(CH_3)_3CSi(C_6H_5)_2O$
2. Boc, *tert*-oxybutylcarbonyl, $C(CH_3)_3OCO–$
3. Strain 3D7 is chloroquine-sensitive.
4. $IC_{50}$ of chloroquine was 12~50 nM, within the expected range for this strain.

treated with **11** was lower relative to the solvent control group on days 4 ($p=0.004$), 5 ($p<0.001$), and 6 ($p=0.008$). The parasitemias were no longer different on day 7. As for quinine, there was almost a significant difference in parasitemia on day 4 ($p=0.064$) and a significant difference on day 5 ($p=0.002$). Parasitemias between the quinine and solvent groups were similar thereafter. There was no significant difference between the parasitemias in the quinine and compound **11** groups on all days scored.

In contrast, the antimalarial compound heptyl prodigiosin (20 mg/kg) retarded parasitemia until day 12 and significantly increased survival time (Fig. 3). (All surviving mice were allowed to eventually die of anemia.)

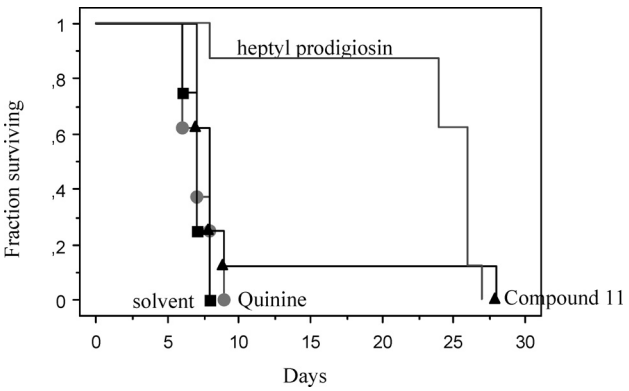


**Fig. 3** Kaplan-Meier survival curves of compound **11** (80 mg/kg), quinine (40 mg/kg), and solvent (80 $\mu$l of 10% DMSO in peanut oil), and antimalarial compound heptyl prodigiosin (20 mg/kg).

Drugs and solvent were administered subcutaneously on day 3 after infection. Number of mice per group=8. Mantel-Cox log-rank $\chi^2=3.361$, $p=0.1863$.

### *In Vitro* Drug Susceptibility Assays on Hepatic Stages of *Plasmodium yoelii yoelii*

The *in vitro* activities of the compounds against hepatic stages were examined using *P. yoelii yoelii* cultured in mouse hepatocytes.

Hepatocytes were collected from the liver of autopsied

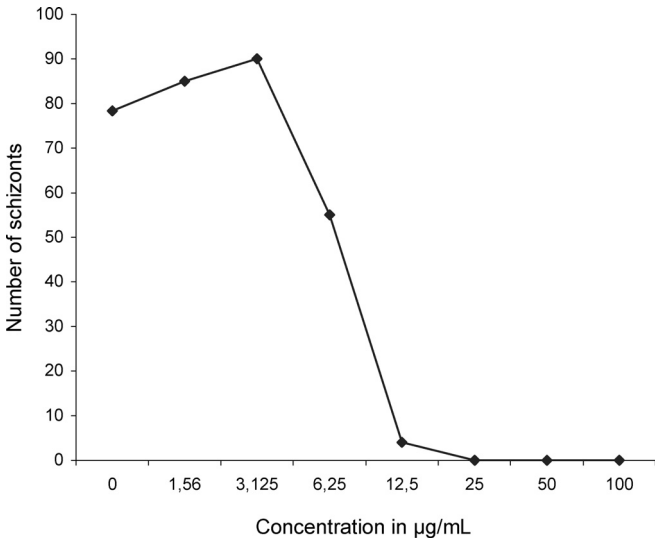


**Fig. 4** Activity of compound **11** on hepatic stages of *P. yoelii yoelii* grown in cultured mouse hepatocytes.

Concentrations are given on a semi-logarithmic scale. $IC_{50}$=8 $\mu$g/ml, or 9.2 $\mu$M.

8～16 week old Swiss mice (Charles Rivers, Orleans, France). They were isolated from the tissues by collagenase perfusion as previously described [11, 12] then resuspended in William's medium (Gibco, Cergy-Pontoise, France) supplemented to a final concentration of 10% fetal calf serum (Gibco), 2% penicillin - streptomycin, 1% sodium pyruvate, 1% L-glutamine, 1% insulin - transferrin - selenium, at 37°C in 4% $CO_2$. Eighty thousand mouse hepatocytes were seeded in each well allowing cell confluence. Cultures were incubated at 37°C, 4% $CO_2$ and 96% air for 24 hours before sporozoite inoculation.

Sporozoites were obtained by dissection of the salivary glands of *Anopheles stephensi* mosquitos infected with *P. yoelii yoelii* (265 BY strain). Sporozoites were suspended in complete William's culture medium and kept chilled until counted in a Malassez chamber.

About $8\times10^4$ sporozoites were applied to each well of mouse hepatocyte cultures. Seven concentrations were tested, ranging between 1 and 100 mg/liter. After 3 hours the culture medium was removed and replaced by fresh medium containing the drug at the selected concentration. Incubation proceeded for 48 hours.

Slides were then fixed in cold methanol for 10 minutes, then incubated for 1 hour at 37°C with a polyclonal antibody directed against the heat shock protein of *Plasmodium* (HSPi72) at a dilution of 1/500 [11, 12]. The slides were then washed in phosphate-buffered saline (PBS) and incubated for 1 hour at 37°C with a solution containing an anti-mouse IgG FITC conjugate (Sigma) diluted at 1/500, with 4′,6-diamidino-2-phenyindole (DAPI) (Sigma) diluted at 1/1000 and Evans Blue (Sigma) at 1/150000. The slides were then mounted with PBS - glycerol (50 : 50, vol/vol) and the schizonts were examined at 250× magnification. The number and size of schizonts in the cultures were noted. For the hepatic stage, the $IC_{50}$ of each compound was determined from the schizont counts calculated from four replicate cultures [12].

**Table 2** Size of schizonts in the treated cultures compared with untreated cultures

| Compounds | Size ($\mu$M) | p |
|---|---|---|
| Control | 19.5±8.57 | |
| Compound **11** | | |
| 6.125 $\mu$g/ml | 3.57±0.1 | <0.0001 |
| 12.5 $\mu$g/ml | 14.8±6.54 | NS |

**Table 3** Antimalarial activity of synthetic analogues against hepatic stages of *P. yoelii yoelii* at 48 hours

| Compounds | Activity against hepatic stages of *P. yoelii yoelii* $IC_{50}$ ($\mu$g/ml)[a] | Effect on schizont size |
|---|---|---|
| **1** | >100 | No |
| **3** | 38.02 | No |
| **6** | >100 | No |
| **8** | 63.81 | No |
| **10** | >100 | No |
| **11** | 8.02 | Yes |
| **12** | 67.8 | No |
| **15** | >100 | No |

[a] $IC_{50}$ were calculated from the average number of schizonts in four replicate wells for 9 concentrations of each drug comparatively to controls.

Compound **11** reduced the number of hepatic schizonts, with an $IC_{50}$ of 8 $\mu$g/ml or 9.2 $\mu$M (Fig. 4). This observation was associated with marked alteration of schizont morphology without alteration of hepatocytes (data not shown). A significant reduction of the mean schizont size was also noted compared to controls (Table 2). Staining with DAPI showed that treated schizonts contained much less nuclear material than control (data not shown).

Compounds **1**, **3**, **6**, **8**, **10**, **12**, and **15** were also tested, but inhibitory effects on the maturation of hepatic stages were moderate (Table 3).

**Table 4** Results of the L5178Y *in vitro* micronucleus assay of compound **11** with S9 activation. The compound is not genotoxic according to this test

| Product | Dose μg/ml | %Survival based on MTT assay | Micronucleated lymphocytes per 2000 cells | $\chi^2$ | p |
|---|---|---|---|---|---|
| DMSO | 0 | 100 | 3 | | |
| Cyclophosphamide | 10 | 101.6 | 79 | 71.913 | <0.001 |
| Compound **11** | 50.0 | 60.6 | 4 | 0 | N.S. |
| | 25.0 | 103.5 | 3 | 0.167 | N.S. |
| | 12.5 | 110.9 | 2 | 0 | N.S. |

**Micronucleus Assay**

A microscale version of the *in vitro* rodent micronucleus assay for DNA fragmentation activity was used as described [13]. Briefly, cells were treated with drug, with or without S9 liver microsomes (S9 mix). Compound **11** was dissolved in DMSO at a concentration of 10 mg/ml, diluted to 1000 μg/ml and then for 10 successive 2-fold dilutions thereafter with Fisher's medium (with 10% decomplemented horse serum, or FM10; Gibco BRL, Paisley, UK) with or without 10% S9 mix. Contents were then dispensed (100 μl) into 96-well plates (Costar, Brumath, France). Mouse lymphoma L5178Y (TK +/− clone 3.7.2C, Porton Down, Salisbury, UK) in 100 μl of culture were added to all wells (600,000 cells/ml for S9 treatment; 650,000 cells/ml for no S9 and 24 hours incubation; 400,000 cells/ml for no S9 and 24 hours incubation plus a 20-hour recovery period).

Cells treated without S9 were cultured with drug for 24 hours (37°C, 5% $CO_2$, 100% humidity). They were then either washed and incubated for a further 20 hours (recovery period) without drug, or harvested immediately. Cells treated in the presence of S9 were cultured for 4 hours with drug, washed to remove S9 and drug, incubated for a further 20 hours, then harvested. Positive controls were cyclophosphamide (10 μg/ml final concentration in wells) for treatment with S9, and mitomycin C (0.05 μg/ml without recovery period or 0.025 μg/ml with a 20-hour recovery period) for treatment without S9. Negative control was 1% DMSO in culture medium.

Treatments were done in duplicate and coupled to a test for cytotoxicity (see below). Harvested cells were fixed and stained. The number of micronuclei-containing cells per 2000 cells was counted in wells that showed at most 70% growth inhibition as determined by the MTT assay. Data were analyzed using the $\chi^2$ ($\alpha$=0.05, degrees of freedom=1) test against DMSO controls.

Compound **11** did not cause DNA fragmentation in this test (Table 4).

**Cytotoxicity Evaluation Using MTT**

For every 96-well plate prepared above, a parallel 96-well plate was prepared to determine cytotoxicity, except that all volumes were halved. Survival estimation based on MTT reduction to formazan was modified from Mossman [14], and carried out at the time of harvesting.

The L5178Y cells were rinsed once then resuspended with 100 μl FM10. To each well was then added 100 μl of 0.5 mg/ml MTT (5 mg/ml MTT in PBS, diluted 10× in Dulbecco's modified Eagle's medium without phenol red), and the cells were incubated for 3 hours (37°C, 5% $CO_2$, 100% humidity). The medium was decanted, and the cells resuspended in acid isopropanol (1 volume 1 N HCl to 23 volumes isopropanol) for 10 minutes with agitation. The plates were read in an ELISA plate reader (550 nm absorbance wavelength, 620 nm reference wavelength).

The percent inhibition was determined relative to DMSO controls, and the $IC_{50}$ determined by linear regression of p′ *versus* log concentration as done for the *in vitro* malaria test. ANOVA was done as described above.

Compound **11** was cytotoxic against L5178Y cells ($IC_{50}$ of 5.8 μM for a 24-hour exposure to the drug, without S9 activation). This $IC_{50}$ is at least 10 times higher than that against malaria.

Compound **11** was clearly an active drug, combining *in vitro* antiparasitic activity with significant reduction in the number and size of schizonts in treated cultures and marked morphological alterations of the parasite. And it appears that the guanidine nucleus and the aliphatic chain are both important for maximum activity.

The ionic pocket in the guanidine nucleus might explain the molecule's biological activity. The guanidinium was found to interact with the fluoroborate anion in a bidentate ligating mode similar to that observed with carboxylates and phosphates [15]. The same kind of hydrogen-bond-

mediated interaction is involved in substrate recognition at enzyme binding sites involving the guanidine-containing side chain of arginine residues, for example. In addition, the guanidinium has been tested in synthetic host receptors for phosphate- and carboxylate-containing host molecules [16].

A long aliphatic chain, on the other hand, appears to improve activity. These results support the suggestion of Overman *et al.* that a long chain might be important for binding to membranes, acting as a chaperone for the guanidino nucleus [17].

Other authors have suggested a role for polyamines. Putrescine linked to chloroquine, for example, was found to increase the drug's activity, perhaps by taking advantage of the increased permeability of the infected erythrocyte to polyamines [18]. In a similar approach, targeting choline transporters was shown by Wengelnik *et al.* to be effective, achieving cures in a primate model with as little as 0.01 mg/kg by intramuscular route and 4 mg/kg by oral route of a choline analogue, G25 [19].

The mechanism of action of compound **11** is not known, although ptilomycalin A was shown to inhibit $Na^+$, $K^+$, and $Ca^{2+}$-ATPases ($IC_{50}$=2～10 $\mu$M) by competitive inhibition at the ATP-binding site [20]. Crambescidin 816 was shown to specifically block L-type calcium channels (with possible consequences on calcium-dependent ATPases), with an $IC_{50}$ of 0.15 nM, 10000 times smaller than the calcium channel blocker nifedipine [21].

In contrast, the antimalarial drug artemisinine appears to bind the plasmodial ATPase PfATP6, but no information on the activity of the compounds described here on artemisinin-resistant strains have been obtained [22].

Compound **11** itself might not be useful as a causal prophylactic. Its $IC_{50}$ (490 nM) is not as low as that of chloroquine (12～50 nM), artemisinin (1～20 nM), halofantrine (0.2～5 nM), and atovaquone (0.2～2 nM). However, the uniqueness of the structure and the possibility of synthesizing analogues could make the molecule interesting as a lead compound.

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