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Growth inhibition of *Plasmodium falciparum* in in vitro cultures by selective action of tryptophan-*N*-formylated gramicidin incorporated in lipid vesicles

Gert N. Moll *, Vincent van den Eertwegh, Huibert Tournois **, Ben Roelofsen, Jos A.F. Op den Kamp and Laurens L.M. van Deenen

Centre for Biomembranes and Lipid Enzymology, University of Utrecht, Utrecht (The Netherlands)

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We studied the differential effect of tryptophan-*N*-formylated gramicidin on uninfected and *Plasmodium falciparum*-infected erythrocytes. Trp-*N*-formylated gramicidin induces a much faster leakage of K^+ from infected cells than from uninfected cells whereas, and at an even lower concentration, gramicidin A' causes a rapid K^+ leakage from both uninfected and infected cells. We also studied the effect of Trp-*N*-formylated gramicidin and gramicidin A' incorporated in liposomes on the growth of *Plasmodium falciparum* in an *in vitro* culture. Incorporation of Trp-*N*-formylated gramicidin in the membranes of so-called 'stealth' vesicles strongly decreases the concentration needed to induce 50% inhibition of parasite growth. Moreover, no decrease in the K^+ content of uninfected cells was observed when cells were exposed to liposome-incorporated Trp-*N*-formylated gramicidin at a concentration which causes full inhibition of parasite growth. These observations strongly suggest that Trp-*N*-formylated gramicidin incorporated in 'stealth' vesicles ends up specifically in the infected cell, thereby inhibiting the growth of the malaria parasite.

Introduction

Linear gramicidins are well known for their antibiotic activity, primarily directed against Gram-positive bacteria, which is related to their ability to selectively facilitate passive diffusion of small cations through membranes [1–3]. The unusually high concentration of tryptophans near the C terminal end of the gramicidin molecule, as well as the role of these tryptophans in channel activity [4,5] and for inhibition of RNA polymerase [6], led Killian et al. [7] to further investigate the importance of the tryptophans by *N*-formylation of the indole residues of the peptide. The resulting Trp-*N*-formylated gramicidin is non-hemolytic, does not [7] or hardly [8] modulate a preexisting lipid organization and has a reduced ionophoretic capacity [9].

In this study we investigated the ability of Trp-*N*-formylated gramicidin to induce K^+ leakage from uninfected and malaria-infected erythrocytes, as well as the effect of the gramicidin derivative on the growth of *Plasmodium falciparum in vitro*. The reasons for studying these effects of Trp-*N*-formylated gramicidin are based on the findings of Elliot et al. [10], which indicate that the channel lifetime of gramicidin increases when lipid bilayer surface pressure decreases. Since the surface pressure in the lipid bilayer of the malaria infected red cell is known to be lower than in uninfected cells [11], one may expect that Trp-*N*-formylated gramicidin channels will show a prolonged life time in the host cell plasma membrane, thus causing an enhanced efflux of K^+.

It has been observed that incorporation of gramicidin S in lipid vesicles reduces its toxicity as measured by the capacity to induce lysis of, and K^+ leakage from, normal red blood cells [12,13]. In order to reduce the toxicity of Trp-*N*-formylated gramicidin in our experiments, we incorporated the peptide into the membranes

* Present address: Unité INSERM 251, Faculté de Medecine X. Bichat, Paris, France.
** Present address: ATO, Agrotechnology, Haagsteeg 6, P.O. Box 17, 6700 AA, Wageningen, The Netherlands.

Correspondence: J.A.F. Op den Kamp, Centre for Biomembranes and Lipid Enzymology, University of Utrecht, P.O. Box 80054, 3508 TB Utrecht, The Netherlands.

of so-called 'stealth' vesicles which – *in vivo* – escape clearance by liver, spleen and macrophages [14]. As it is known that interactions of malaria infected erythrocytes with lipid vesicles and subsequent migration of phospholipids across the erythrocyte plasma membrane into the parasite occur [15], the additional advantage of the use of such vesicles is that it will facilitate the specific transfer of Trp-*N*-formylated gramicidin towards the infected cells. We studied firstly whether Trp-*N*-formylated gramicidin incorporated into vesicles acts selectively on infected cells, and secondly, since indications exist that potassium is required for the growth of the parasite [16,17], the capacity of Trp-*N*-formylated gramicidin to inhibit the growth of *Plasmodium in vitro*.

Materials and Methods

Chemicals

Gramicidin A' (80% gramicidin A, 4% gramicidin B, 16% gramicidin C), distearoylphosphatidylcholine, monosialoganglioside (GM1) and cholesterol were obtained from Sigma (St. Louis, MO, U.S.A.). ^{14}C-labeled gramicidin (specific activity 57 mCi/mmol) was prepared according to Tournois et al. [18]. Trp-*N*-formylated gramicidin and ^{14}C-labeled Trp-*N*-formylated gramicidin (specific activity 0.272 mCi/mmol) were prepared by formylation of the four tryptophan residues according to Classen et al. [9]. [G-^{3}H]Hypoxanthine was obtained from Amersham International, U.K. The growth medium for culturing *P. falciparum*, RPMI 1640, was obtained from Gibco Europe, Breda, The Netherlands. Blood and AB^{+} serum were obtained from the local blood bank.

Production and collection of Plasmodium falciparum infected cells

P. falciparum was cultured according to Jensen and Trager [19]. Uninfected and infected cells were separated by percoll-sorbitol density gradient centrifugation according to Kutner et al. [20].

Preparation of gramicidin dispersions and incorporation of gramicidin into vesicles

Since neither gramicidin nor Trp-*N*-formylated gramicidin are easily water soluble [21], both compounds were dispersed in isotonic buffer, prior to their addition to the cell suspensions. The concentration of [^{14}C]gramicidin (specific activity 57 mCi/mmol gramicidin A' and 0.272 mCi/mmol Trp-*N*-formylated gramicidin) was determined on the basis of the radioactivity which was measured using 299 TM emulsifier scintillation solution from Packard, in a Packard scintillation analyzer TRI CARB 1500.

Gramicidin containing stealth vesicles, consisting of distearoylphosphatidylcholine, monosialoganglioside, cholesterol and gramicidin in molar ratios of 10 : 1 : 5 : 0.5, were prepared by injection of 150 µl of an ethanolic solution of the above mentioned components (containing 3–13 mM distearoylphosphatidylcholine) into 3 ml RPMI 1640 [22]. The thus prepared vesicles were diluted to such extent that, upon addition to the *Plasmodium* culture, final ethanol concentrations of less than 0.07% v/v were obtained. In the case of survival experiments, we also used vesicles of the same composition, but prepared by sonication [15], giving identical concentrations needed to induce 50% inhibition of parasite growth in the case of Trp-*N*-formylation and very close values in the case of gramicidin A'.

Measurement of K^{+} efflux

In order to measure the gramicidin induced K^{+} leakage from uninfected and infected erythrocytes, 20 µl erythrocytes (approx. 80 nmol phospholipid) were washed in an isotonic solution of choline chloride, buffered with 0.1 mmol/l imidazole (pH 7.4) and resuspended in 5 ml of the same buffer. The choline chloride buffer was prepared by using demineralized and double distilled water. This buffer was also used to prepare gramicidin dispersions and gramicidin containing vesicles for determining their effects on K^{+} efflux from cells, which was measured continuously up to 5 min using a K^{+} sensitive glass electrode (Philips type: 15K) and a reference electrode (Philips type: R44/2-SD/1). K^{+} leakage proceeded linearly during this period in all cases tested. The addition of Triton X-100 allowed the measurement of the total K^{+} content of the sample. K^{+} leakage is expressed as % of total cellular K^{+} released per min. Long incubations of cells were impossible due to the fragility of both uninfected and infected erythrocytes.

Survival assay

The effect of gramicidin A' and its derivative on the growth of *Plasmodium falciparum* was studied by determining parasite survival [11], using the method originally described by Desjardin [23]. *P. falciparum* was cultured in microwell plates essentially according to Jensen and Trager [19], cultures were not synchronised. Experiments were started by the addition of various volumes of a dispersion of ^{14}C-labeled gramicidin to 200 µl erythrocyte suspension (2% v/v erythrocytes, 1% parasitized) in RPMI 1640 containing 10% AB^{+} serum. The exact amount of [^{14}C]gramicidin added was determined on the basis of its radioactivity as described above. After 24 h of incubation, 0.5 µCi [G-^{3}H]hypoxanthine (8.6 Ci/mmol) was added and after another 24 h cells were lysed and the ^{3}H-labeled nucleic acids were recovered on a filter using a cell harvester (Nunc). Survival was calculated on the basis of the [^{3}H]hypoxanthin incorporation in nucleic acids.

Results and Discussion

Because a decreased surface pressure causes an extension of the lifetime of a gramicidin channel [10], whereas the surface pressure in the plasma membrane of malaria-infected red cells is known to be decreased [11], we first studied whether there might be a differential effect of gramicidin A' and Trp-*N*-formylated gramicidin on the K^+ leakage from infected erythrocytes when compared to uninfected cells. Since we measured a marked decrease in K^+ content of infected cells when compared to uninfected cells – which is in agreement with other studies [24,25] – it is necessary to relate the extent of K^+ leakage to the total K^+ content of the very same sample of cells. We observed that the K^+ leakage from erythrocytes (either or not infected) is a linear process. Consequently, the data in Table I are expressed in % of total cellular K^+ released per min. No K^+ leakage was observed when cells – either uninfected or infected – were incubated in the absence of gramicidin. At a concentration of 60 nM, gramicidin A' induces a slightly faster K^+ leakage from infected (7.4%/min) than from uninfected erythrocytes (6.0%/min) (Table I). Trp-*N*-formylated gramicidin is much less effective, as an appreciably higher concentration (200 nM) is needed to induce a detectable rate of K^+ leakage from uninfected cells (Table I). A strong decrease in the gramicidin A' induced K^+ leakage rate from uninfected cells is observed when the drug is incorporated in vesicles (Table I). In contrast to uninfected cells, however, infected cells rapidly leak K^+ upon addition of vesicle-incorporated gramicidin A'. Similar observations were made with Trp-*N*-formylated gramicidin, a decrease in K^+ leakage rate from uninfected cells is observed upon its incorporation in the vesicles. The capacity to induce

TABLE I

K^+ efflux from uninfected and malaria infected erythrocytes induced by gramicidin A' and Trp-N-formylated gramicidin

P. falciparum was cultured according to Jensen and Trager [19]. Uninfected and malaria infected erythrocytes were separated by percoll-sorbitol density gradient centrifugation according to Kutner et al. [20]. Intracellular K^+ leakage from uninfected and malaria-infected erythrocytes, induced by gramicidin A' or Trp-*N*-formylated gramicidin, was measured using a K^+ sensitive glass electrode (Philips, type: 15 K) and a reference electrode (Philips, type: R44/2- SD/1). Data are duplicate measurements of representative experiments. See Materials and Methods for further details.

		K^+ leakage rate (%/min)	
		uninf. cells	inf. cells
Gramicidin A': dispersions	(60 nM)	6.0 ± 0.8	7.4 ± 0.4
Gramicidin A': in vesicles	(200 nM)	1.8 ± 0.4	12.4 ± 2.4
Trp-*N*-formylated gramicidin: dispersions	(200 nM)	1.4 ± 0.2	7.2 ± 1.2
Trp-*N*-formylated gramicidin: in vesicles	(200 nM)	0.4 ± 0.2	3.4 ± 0.4

TABLE II

Inhibition of the growth of P. falciparum by gramicidin A' and Trp-N-formylated gramicidin

Concentrations of gramicidin A' and Trp-*N*-formylated gramicidin causing 50% growth inhibition (IC_{50}) of *Plasmodium falciparum* were determined as described in Materials and Methods. Values represent the means (± S.D.) of triplicate measurements performed on samples derived from three independent experiments, except in the case of Trp-*N*-formylated gramicidin dispersions where a range of concentrations is given based on four independent experiments. Vesicles without gramicidin did not inhibit parasite growth.

	Concentrations causing 50% growth inhibition (nM)	(*n*)
Gramicidin A': dispersions	4.40 ± 1.2	(3)
Gramicidin A': in vesicles	0.80 ± 0.07	(3)
Trp-*N*-formylated gramicidin: dispersions	565–17500	(4)
Trp-*N*-formylated gramicidin: in vesicles	6.99 ± 0.55	(3)

K^+ leakage from uninfected cells decreases in the following order: gramicidin A' > gramicidin A' incorporated in vesicles ≥ Trp-*N*-formylated gramicidin > Trp-*N*-formylated gramicidin incorporated in vesicles (Table I). In contrast to gramicidin A' dispersions, Trp-*N*-formylated gramicidin dispersions induce a much faster K^+ leakage from infected cells (7.2%/min) than from uninfected cells (1.4%/min) (Table I). Furthermore, plotting $1/(K^+$ leakage) versus log(Trp-*N*-formylated gramicidin) produces a straight line for both uninfected and infected cells (not shown), from which it can be calculated that uninfected cells require eight times higher concentrations of the derivatized drug than infected cells to undergo the same rate of K^+ leakage. Vesicle-incorporated Trp-*N*-formylated gramicidin induces a very low rate of K^+ leakage from uninfected cells (0.4%/min), whereas it is still able to induce a considerable K^+ leakage rate from infected cells (3.4%/min) under the same conditions.

Having observed a differential effect of Trp-*N*-formylated gramicidin on infected and uninfected cells, it is of considerable interest to study its possible effect on the growth of *P. falciparum in vitro*. To this end, we used a previously described technique [13,23]. *Plasmodium falciparum* was grown for 24 h in the presence of various concentrations of either gramicidin A' or Trp-*N*-formylated gramicidin. Inhibition of the growth of *Plasmodium* was calculated on the basis of [^{3}H] hypoxanthin-incorporation in the parisite's nucleic acids during a subsequent 24 h of cultivation. Addition of gramicidin A' dispersions to a suspension of mixed uninfected and infected cells (99 : 1) shows 50% inhibition of parasite growth (IC_{50}) at a concentration of 4.40 ± 1.2 nM (Table II). When gramicidin A' is incorporated in the vesicles, a 5-fold lower IC_{50} value is measured. However, a most dramatic decrease in IC_{50}

value is observed following the incorporation in vesicles of Trp-*N*-formylated gramicidin. In the case of dispersions of Trp-*N*-formylated gramicidin, the IC_{50} value varies between 565 and 17500 nM, but when incorporated in the vesicles, as little as 6.99 ± 0.55 nM of Trp-*N*-formylated gramicidin is sufficient to cause 50% inhibition.

The above survival experiments start off with a suspension of 1% parasitized erythrocytes. This implies that, when a gramicidin dispersion is added to the cell suspension, most – if not 99% – of the gramicidin will enter the plasma membrane of uninfected cells. The observation that 5-times less gramicidin A′ and much (at least 80-times) less Trp-*N*-formylated gramicidin is sufficient for 50% inhibition of parasitic growth when these antibiotics are incorporated in the membranes of the lipid vesicles, strongly suggests that these conditions greatly favour the incorporation of the drugs – and most specifically that of Trp-*N*-formylated gramicidin – into the infected cells. On one hand, incorporation of the gramicidin molecules in vesicles most likely counteracts their entrance into the plasma membrane of uninfected erythrocytes as evidenced by the reduction of the gramicidin induced K^+ leakage from these cells (Table I). On the other hand, however, the fact that infected erythrocytes can take up phospholipids when extracellularly offered as vesicles [15], and the fact that the plasma membrane of infected cells has a notably decreased surface pressure [11], will favour the migration of the gramicidin molecules from the vesicles to the infected erythrocytes.

In order to investigate whether under culture conditions the vesicle-incorporated gramicidins, when administered to a mixed population of uninfected and infected cells also affect uninfected erythrocytes, suspensions of erythrocytes (7% haematocryt, 1.2% parasitemia, total volume 1.5 ml) were submitted to a range of concentrations of the vesicle-incorporated gramicidins. Upon 12 h of incubation, parasitemia increases up to 2% in control samples, whereas parasites have disappeared from the cultures that had been supplemented with either 6.66 nM vesicle-incorporated gramicidin A′ or 66.6 nM vesicle-incorporated Trp-*N*-formylated gramicidin, as observed by microscopic inspection of Giemsa stained smears. In either of these two cases, the K^+ content of the remaining uninfected cells, determined after washing the cells with isotonic choline chloride buffer, is identical to the potassium content of isolated uninfected cells from control cultures. This is in line with the notion that the vesicle-incorporated gramicidins preferentially enter the infected cells.

The question now to be addressed is whether the gramicidins incorporated in vesicles also inhibit the growth of *Plasmodium in vivo*. In order to approach the *in vivo* situation, fresh human heparinized blood was mixed with *P. falciparum* infected red blood cells (blood group O), giving an initial parasitemia of 0.7%. Upon 8 h of incubation at 37°C under gentle rotation in sealed and completely filled cups, in the presence of various concentrations of vesicle-incorporated gramicidin A′ or vesicle-incorporated Trp-*N*-formylated gramicidin, the parasitemia was determined by inspection of Giemsa-stained smears. Parasitemia remains constant in the absence of gramicidin, but a marked concentration dependent decrease in parasitemia is observed in the presence of either gramicidin A′ or Trp-*N*-formylated gramicidin. Vesicle-incorporated gramicidin A′ and vesicle-incorporated Trp-*N*-formylated gramicidin cause a 50% disappearance of parasites when they are present at concentrations of 0.29 μM and 1.08 μM, respectively. It should be noted that *in vitro* the parasites do not grow in total blood [16] which precludes incubations for longer time periods. Since we were unable to detect any hemolytic effect of Trp-*N*-formylated gramicidin on infected erythrocytes during relatively short incubations of pure infected cells with Trp-*N*-formylated gramicidin, presumably the disappearance of the infected cells results from effects secondary to the killing of the parasite by Trp-*N*-formylated gramicidin.

It is important to note observations by Ginsburg et al. [24], implying that, especially in late stages of infection, the K^+/Na^+ ratio in the space between the plasma membrane and the parasite surrounding vacuolar membrane approaches a value of 0.8, whereas inside the parasite surrounded by the – second intraerythrocytic – parasite membrane this ratio can be as high as 5. This might have major consequences for the evaluation of the data on the differential efflux of K^+ from uninfected and infected cells as brought about by Trp-*N*-formylated gramicidin (Table I) and likewise for the evaluation of the K^+ leakage induced by gramicidin A′. Since we observe that K^+ leakage proceeds according to one phase kinetics (not shown), Trp-*N*-formylated gramicidin should have entered the infected cell where it enables efflux of intraparasitic K^+. Channels should therefore be formed, not only in the host cell plasma membrane, but also in the vacuolar and parasite's membrane, thus permitting the release of intraparasitic K^+.

Gramicidin A′ is known as an antibiotic that is toxic to man, although it is used for external applications. Its high toxicity is mainly ascribed to its capacity to enhance membrane permeability, resulting in the uncoupling of oxidative phosphorylation and hemolysis of red blood cells. These characteristics strongly depend on the C terminal tryptophans in the peptide. Upon Trp-*N*-formylation, the molecule is no longer hemolytic and the channel forming ability is reduced. Trp-*N*-formylated gramicidin will therefore be much less toxic than gramicidin A′. Incorporation of Trp-*N*-formylated gramicidin into the lipid vesicles results in a further reduction of toxicity. These aspects, as well as the present observation that vesicle-incorporated Trp-*N*-formylated grami-

cidin specifically attacks the infected erythrocytes, challenge us to further study the toxicity of this system and its efficiency in inhibiting malarial growth *in vivo*.

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