Cytotoxicity of *N*-dodecanoyl-L-homoserine lactone and 5-*N*-dodecyl resorcinol to human granulocytes and monocytes: a comparative study

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Abstract

Small molecules originating from microbes (SMOMs) play a significant role in bacterial-bacterial communication and in interactions between bacteria and their hosts. The aim of this study is a comparative analysis of the cytotoxic activity to human granulocytes and monocytes of two structurally close SMOMs: N-dodecanoyl-L-homoserine lactone (C_{12}-HSL) and 5-N-dodecyl resorcinol (C_{12}-AR). Cell viability was determined by Trypan blue dye exclusion. To distinguish cell death mechanisms, both necrosis and apoptosis tests were carried out. Cells undergoing apoptosis were identified by caspase-3 activity and the level of histone-associated DNA fragments. To evaluate cell lysis, the lactate dehydrogenase release test was used. In addition, the SMOM's action on erythrocyte membrane stability was investigated.

The investigated SMOMs in micromolar concentrations showed more dose-dependent cytotoxicity against granulocytes than monocytes, but they used different mechanisms to impinge on the cell death pathway. C_{12}-HSL specifically induced apoptosis similar to the activity previously reported for 3-oxo-N-dodecanoyl-L-homoserine lactone that originated from Pseudomonas aeruginosa. In contrast, C_{12}-AR induced fast cytolytic effects (necrosis) as shown by the release of cytoplasmic lactate dehydrogenase, and presumably were defined by cellular membrane destabilisation.

Our data demonstrate that both C_{12}-HSL and C_{12}-AR can eliminate key defence cells, which would otherwise participate in the destruction of pathogenic bacteria. These results reinforce the SMOMs bifunctionality concept that such bacterial molecules not only regulate bacterial-bacterial interactions but also break immune defences as a new and important mechanism of host evasion.

Key words: apoptosis, cytotoxicity, necrosis, N-dodecanoyl-L-homoserine lactone, 5-N-dodecyl resorcinol.

Introduction

Small molecules originating from microbes (SMOMs) play a significant role in bacterial-bacterial communication and also in inter-kingdom signalling: interactions between bacteria and their hosts [1]. Examples of SMOMs are *N*-acyl homoserine lactones (HSLs) and resorcinolic lipids (RLs); they vary in the structure of the nonpolar *N*-acyl chain and polar lactone or resorcinol single-ring. The HSLs are involved in quorum sensing, which is a mechanism of bacterial gene expression control in response to increasing cell density [2]. The RLs are synthesised by some pro- and eukaryotes [3] and operate as inductors of bacterial cell transition into the dormant stage [4]. On the other hand, these amphiphilic substances diffuse easily through epithelial barriers into human liquids and tissues where experimentally detected HSLs and RLs concentrations vary from nano- to micro-molar [5, 6]. As a result, HSLs affect the function of a wide range of mammalian cell types (including cells of the immune system), repress the production of

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cytokines, disrupt the function of antigen-presenting cells [7, 8], and alter cell metabolism and cycling [9]. In turn, RLs could act in the human body as antioxidant, antigenotoxic, and cytostatic agents [10].

An extreme SMOMs biological activity is a cytotoxic effect potentially developing in two ways: necrosis and apoptosis. Mammalian cells undergoing necrosis typically lose membrane integrity, shut down metabolism and release their contents into the environment [11]. In contrast, apoptotic cells do not lyse but activate specific energy-dependent mechanisms that lead to condensation of chromatin and cleavage of DNA into regularly sized fragments [12]. Cytotoxic activities have been shown for different HSLs [13, 14] or RLs [15, 16] in some cases observed by the induction of apoptosis, and can be explained by their interactions with DNA and membrane-associated targets. However, the similarities or distinctions of these small molecules in cytotoxicity are not completely clear.

The aim of this study is a comparative analysis of the cytotoxic mechanisms of two structurally close HSL and RL: *N*-dodecanoyl-L-homoserine lactone and 5-*N*-dodecyl resorcinol in human granulocytes and monocytes.

Material and methods

Chemicals

N-dodecanoyl-L-homoserine lactone (C_{12}-HSL) was purchased from Cayman Chemical Company (Michigan, USA). 5-*N*-dodecyl resorcinol (C_{12}-AR) was synthesised according to standard organic procedures and purified to 99% homogeneity by preparative liquid chromatography by Enamine Ltd. (Kiev, Ukraine). These compounds have a similar acyl chain but typical of HSLs and ARs polar head groups, respectively (Fig. 1).

C_{12}-HSL and C_{12}-AR stock solutions (0.01 M) were prepared in 96% ethanol, and diluted in Hank's balanced salt solution (HBSS) or 0.85% NaCl solution containing 5% ethanol immediately before use. The basic concentrations varied from 10^{-4} M to 10^{-7} M, which corresponded to the experimentally-detected presence of HSLs and RLs in biological liquids and tissues of humans [5, 6].

Cell culture and treatments

Human granulocytes and monocytes were isolated from leukocyte-rich plasma originating from heparin-treated peripheral blood < 2 h old. The plasma samples were put on a double ficoll-verografine density gradient (1.077 and 1.092 g/ml), and were centrifuged at 800 × g for 15 min at 24 ±4°C. The cells that had been separated on high (monocytes) and low (granulocytes) density barriers were gently washed with cold HBSS and resuspended in Medium 199 to concentrations of $1.05 \pm 0.12 \times 10^6$ for granulocytes and $1.73 \pm 0.15 \times 10^6$ for monocytes, which reflect the physiological leukocytes parities.

The 900 µl granulocyte or monocyte suspensions were plated in wells containing 100 µl C_{12}-HSL and C_{12}-AR dilutions or an equal HBSS volume (control); they were then incubated in a humidified atmosphere containing 5% CO_2 at 37 ±1°C. After 1 to 4 h of incubation, the probes were centrifuged at 1,000 × g for 5 min, and cells or supernatants were tested in different cytotoxicity assays.

For hypotonic stress experiments, cells originating from heparin-treated peripheral blood erythrocytes were washed three times with a 0.85% NaCl solution, resuspended to 108 cells/ml, and pre-incubated with C_{12}-HSL and C_{12}-AR dilutions at 37°C for 1 h.

Cytotoxicity assays

Cell viability test by Trypan blue dye exclusion: The cells treated with or without C_{12}-HSL and C_{12}-AR were collected at the indicated time point and were mixed 1 : 1 with 0.001 M Trypan blue (TB) solution in PBS. The cell colouring and counting were examined by light microscopy in a hemocytometer. The percentage of TB(+) dead cells stained blue due to vital dye was calculated by comparison to that of non-treated control granulocyte and monocyte suspensions.

Quantitation of apoptosis markers: To evaluate the induction of apoptosis, caspase-3 activity and the level of histone-associated DNA fragments were determined in cells treated with C_{12}-HSL and C_{12}-AR.

The quantitative presence of active caspase-3 was measured using the Quantikine ELISA kit (R&D Systems, Minneapolis, USA). After centrifuge preparations, cells were lysed by denaturing extraction buffer containing biotinylated caspase inhibitor diluted 10-20 fold with a calibrator diluent. Caspase-3 activity in cell extracts was determined using a caspase-specific monoclonal antibody coated on the microplate and horseradish peroxidase conjugated to streptavidin that binds to the biotin on the caspase-attached
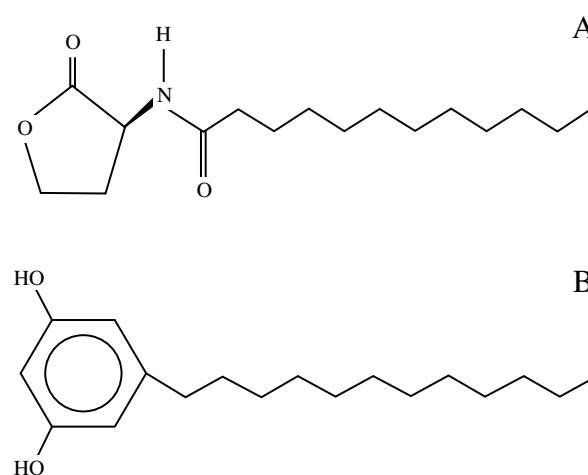
Fig. 1. Structures of C_{12}-HSL (A) and C_{12}-AR (B) used in this study

inhibitor. Calculations were compared to a standard curve and presented as the caspase-3 concentration (ng/ml) of each sample multiplied by the diluting factor.

DNA fragmentation and nuclear membrane breakdown in the C_{12}-HSL and C_{12}-AR treated cells were quantified by the measurement of cytoplasmic mono- and oligonucleosome DNA fragments using the Cell Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Fixed on the microplate wall, mouse monoclonal antibodies specific to the H1-H4 histones and conjugated with horseradish peroxidase anti-DNA antibodies, were used in this sandwich-enzyme-immunoassay. Specific enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated using the following formula: enrichment factor (EF) = A_{405} treated cells/A_{405} control cells.

Cells lysis assays: To evaluate cell membrane integrity, the lactate dehydrogenase (LDH) release test was used. The colorimetric measurement of LDH activity in C_{12}-HSL- and C_{12}-AR-treated granulocyte and monocyte supernatants was performed using the LDH-UV-Novo kit (Vector-Best, Novosibirsk, Russia) according to the manufacturer's instructions. The reduction of pyruvate to lactate in the presence of NADH was monitored kinetically at 340 nm by the rate of decrease in absorbance (Δ), and enzyme activity (EA) was calculated as Δ/min × 6.592.

In a separate experiment, the action of C_{12}-HSL and C_{12}-AR on erythrocyte membrane stability was investigated [17]. The 50-µl aliquots of treated and control samples were added to tubes containing 5 ml of 0.40-0.85% NaCl solutions, gently mixed, and incubated at 37°C for 30 min before being centrifuged at 1500 × g for 15 min. Erythrocyte lysis was followed by measuring the absorbance of the supernatants at 540 nm (A_{540}) and was presented as percentage of haemolysis depending on NaCl content. In turn, these data were used for the calculation of integral membrane stability parameters, which was evaluated by the half-transition point (X_{50}) obtained from the curves of haemoglobin efflux induced by the increase in medium hypotonicity.

Statistical analysis

Data are reported as ± standard errors of the mean of determinations performed in triplicate on three different samples and were analysed by the Student's *t*-test. A *P* value of less than 0.05 was considered significant. Calculations were performed with Statistica V8 for Windows (Stat Soft Inc., Tulsa, USA).

Results

Loss of granulocyte and monocyte viability caused by C_{12}-HSL and C_{12}-AR

C_{12}-HSL and C_{12}-AR had similar effects evaluated by the cell viability assay, such as the uptake of a dye (Trypan

blue) by dead cells after breakdown of the cellular permeability barrier. The cytotoxicity of both compounds was concentration-dependent with varying effects depending on the cell type used (Table 1).

The counting of stained granulocytes demonstrated about 1.4 ±0.8% of TB(+) cells in control samples. However, in C_{12}-HSL (10^{-6}, 10^{-5}, 10^{-4} M) treated samples, the percentage of TB(+) cells increased gradually to 34.0 ±1.84%, 92.1 ±4.88%, and 96.1 ±4.99%, respectively ($p \leq 0.01$). C_{12}-AR cytotoxicity was detected at concentrations of 10^{-5} and 10^{-4} M with 68.6 ±3.49% and 95.9 ±4.98% of TB(+) cells, respectively ($p \leq 0.001$). The lowest C_{12}-HSL and C_{12}-AR concentrations did not induce cytotoxicity.

The toxicity of C_{12}-HSL and C_{12}-AR to monocytes also developed in a concentration-dependent manner but had low intensity. Statistically significant activity was detected in concentrations of 10^{-5} and 10^{-4} M only where the percentages of C_{12}-HSL-treated TB(+) cells were 10.6 ±0.51% and 14.8 ±0.91% ($p \leq 0.05$), respectively, and the percentages of C_{12}-AR-treated cells were 9.8 ±0.55% and 10.9 ±0.61% ($p \leq 0.05$), respectively. The differences in monocyte viability at the lowest C_{12}-HSL and C_{12}-AR concentrations were non-significant compared to controls: 2.2 ±0.13% TB(+) cells.

It is important to note that distinct forms of cell death cannot be distinguished by the trypan blue exclusion assay. This necessitates using specific methods for the detection of apoptotic and necrotic markers in C_{12}-HSL- and C_{12}-AR-treated cells.

Expression of apoptotic markers in C_{12}-HSL- and C_{12}-AR-treated cells

Granulocytes and monocytes were treated with or without C_{12}-HSL or C_{12}-AR for 1 and 4 h. Cell lysates were collected and examined for caspase-3 activity, and the level of histone-associated DNA fragments were compared to the non-treated controls (Table 1). C_{12}-HSL, but not C_{12}-AR, induced apoptosis in a concentration- and time-dependent manner, and the expressiveness of this effect was greater in granulocyte than monocyte cell lines.

We observed that granulocytes treated with C_{12}-HSL had more caspase-3 activity; 10^{-6} M of this compound was enough, and induction of apoptosis started within 1 h. As shown in Fig. 2A, treatment of granulocytes with C_{12}-HSL in concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M for 1 h resulted in a dose-dependent increase in caspase-3 activity to 8.60 ±0.36, 27.26 ±5.34 and 101.27 ±10.34 ng/ml, respectively. A 4-h treatment led to a 4-12-fold increase in caspase-3 activity becoming extremely high (110.35 ±6.38 – 115.35 ±6.70 ng/ml; $p \leq 0.001$) in the cell lysates. The monocyte line was not very sensitive, requiring C_{12}-HSL concentrations of 10^{-5} and 10^{-4} M to increase caspase-3 activity only to 6.95 ±0.39 and 10.25 ±0.58 ng/ml after a 1-h treatment ($p \leq 0.01$), and to 9.05 ±0.53 and 12.05 ±0.75 ng/ml after

Table 1. Effects of C_{12}-HSL and C_{12}-AR on granulocyte (G) and monocyte (M) viability markers after incubation for 1 h

Type of SMOMs	Concentration, M	TB(+) dead cells, %		Concentration of active caspase-3, nM		Enrichment factor, A_{405}		LDH activity, EA	
		G	M	G	M	G	M	G	M
C_{12}-HSL	10^{-7}	5.00 (0.30)	6.20 (0.39)	0.09 (0.01)	1.03 (0.06)	0.87 (0.04)	1.05 (0.06)	712.97 (37.07)	811.46 (47.06)
	10^{-6}	34.00** (1.84)	8.00 (0.45)	8.60** (0.36)	1.04 (0.06)	1.40 (0.09)	1.07 (0.05)	717.24 (32.28)	811.46 (46.25)
	10^{-5}	92.10*** (4.88)	10.60* (0.51)	27.26** (5.34)	6.95** (0.39)	2.43* (0.13)	1.36 (0.11)	713.38 (39.95)	818.27 (45.00)
	10^{-4}	96.13*** (4.99)	14.80* (0.91)	101.27*** (10.34)	10.25** (0.58)	7.03** (0.42)	1.62* (0.09)	726.59 (36.33)	830.95 (44.04)
C_{12}-AR	10^{-7}	6.70 (0.41)	0.01 (0.001)	0.13 (0.01)	0.77 (0.05)	1.05 (0.06)	0.92 (0.05)	694.36 (37.00)	787.24 (43.74)
	10^{-6}	8.50 (0.46)	6.80 (0.42)	0.09 (0.01)	0.79 (0.04)	0.98 (0.05)	1.01 (0.05)	880.59* (46.67)	799.86 (36.79)
	10^{-5}	68.60** (3.49)	9.80 (0.55)	0.13 (0.01)	0.85 (0.06)	1.00 (0.06)	0.84 (0.05)	1041.29** (62.48)	944.68* (57.63)
	10^{-4}	95.90*** (4.98)	10.90* (0.61)	0.16 (0.02)	0.89 (0.06)	1.04 (0.06)	0.86 (0.04)	1205.16** (65.08)	959.83* (52.79)
Control	0	1.40 (0.80)	2.20 (0.13)	0.12 (0.01)	1.18 (0.12)	1.00 (0.10)	1.00 (0.09)	701.87 (35.34)	749.73 (41.31)

Data in triplicate on three different samples (± standard errors)

* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001 compared with the non-treated cells
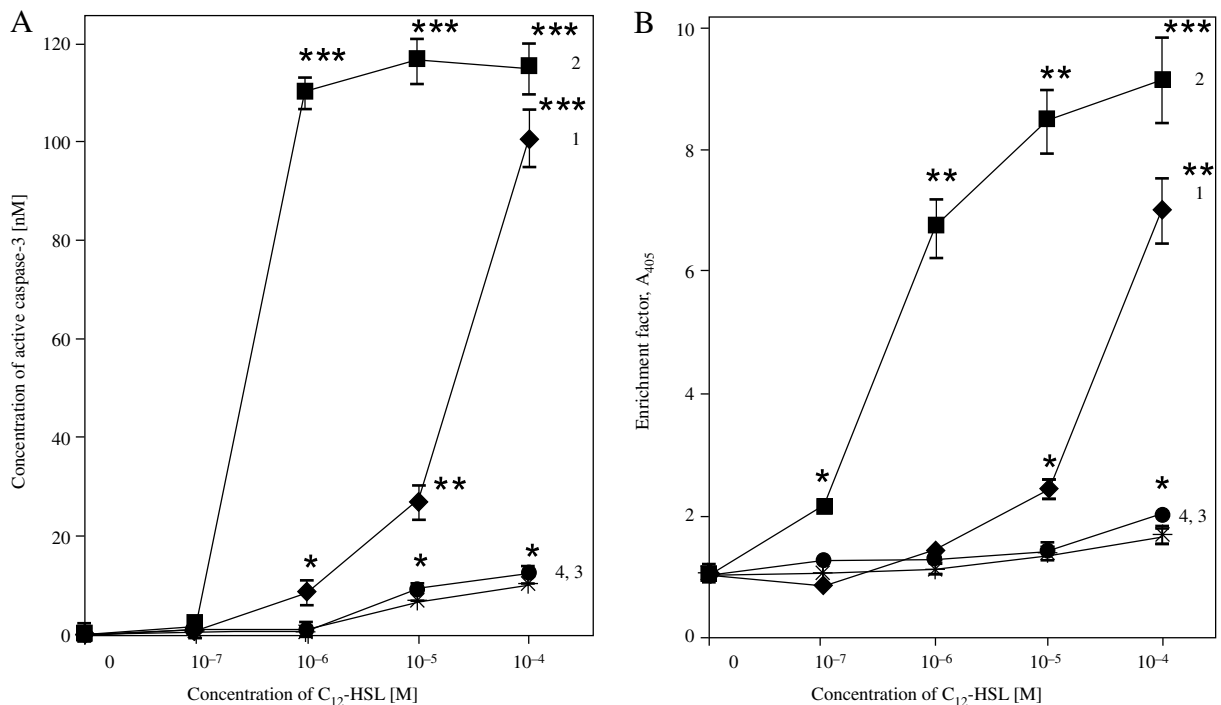
Fig. 2. Changes in apoptotic markers: caspase-3 activity (A) and level of histone-associated DNA fragments (B) after 1- and 4 h-incubations of granulocytes (1, 2) and monocytes (3, 4) with different C_{12}-HSL concentrations. **P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 compared with the nontreated cells*

a 4-h treatment ($p \leq 0.01$), respectively. In the same assay, C_{12}-AR did not show statistically significant activity.

Another hallmark of apoptosis is double-stranded, internucleosomal cleavage of DNA into regularly sized fragments. As shown in Fig. 2B, measurement of histone-associated mono- and oligonucleosome DNA fragments in sandwich-enzyme immunoassays indicated that treatment of granulocytes with C_{12}-HSL led to DNA cleavage within 1 h. A statistically significant enrichment factor (EF) value resulted from concentrations of 10^{-5} and 10^{-4} M: 2.43 ±0.12 and 7.03 ±0.42 ($p \leq 0.01$), respectively. Treatment of granulocytes for 4 h resulted in more intensive DNA fragmentation with EF values from 6.74 ±0.35 to 9.16 ±0.53 ($p \leq 0.01$), and 10^{-6} M C_{12}-HSL was enough to induce apoptotic cell death. We observed similar tendencies when monocytes were treated with C_{12}-HSL, but in this cell line there was less DNA fragmentation.

Together, these results indicate that treatment of granulocytes and monocytes with C_{12}-HSL led to their apoptotic demise correlated with an effect on cell viability as determined using the Trypan blue dye exclusion method. However, these assays were ineffective in revealing C_{12}-AR cytotoxic mechanisms other than apoptosis.

Lactate dehydrogenase release from C_{12}-HSL- and C_{12}-AR-treated cells

To explore the necrotic mechanism of cell death, we examined lactate dehydrogenase (LDH) release from gran-

Fig. 3. Osmotic stability curves of erythrocytes treated with C_{12}-AR in concentrations of 10^{-4} M (1), 5×10^{-5} M (2), 2.5×10^{-5} M (3), and control (4), used for half-transition point (X_{50}) calculation

ulocytes and monocytes treated with or without C_{12}-HSL or C_{12}-AR. This enzyme is normally in the cytoplasm of eukaryotic cells, and its release into the culture medium strongly correlates with cell lysis [18].

Differences in LDH release between C_{12}-HSL treated for 1 h and control cells were not apparent (Table 1). Detectable LDH activity appeared after 4 h of treatment only, probably as a secondary lysis of apoptotic cells (data not shown). In contrast, a significant increase in enzyme activity was demonstrated in cell supernatants treated with C_{12}-AR for 1 h, which is indicative of fast cell lysis. LDH release following treatment with C_{12}-AR was slightly higher in granulocytes (880.59 ±46.67, 1041.29 ±62.48, and 1205 ±65.08; $p \leq 0.01$) than in monocytes (944.68 ±57.63 and 959.83 ±52.79; $p \leq 0.05$), in which high concentrations of this compound were needed.

Taken together, these data suggest that C_{12}-AR induces primary necrosis of granulocytes and monocytes, but C_{12}-HSL contributes to their secondary necrosis as a result of apoptotic cell death.

C_{12}-HSL and C_{12}-AR influence on membrane stability
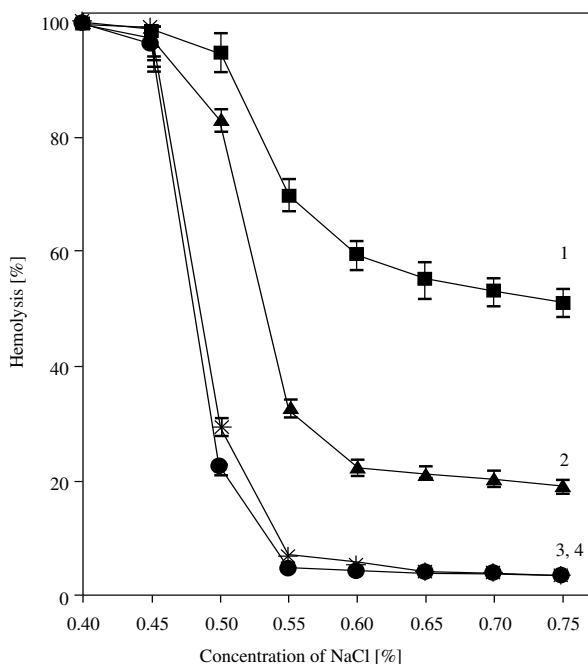
The fast cell lysis by C_{12}-AR, but not C_{12}-HSL, prompted us to examine the influence of these compounds on cell membrane stability. To this end, a hypotonic lysis of erythrocytes pre-incubated with C_{12}-HSL and C_{12}-AR dilutions at 37˚C for 1 h was examined in different NaCl concentrations.

The study revealed no differences between C_{12}-HSL treated and control erythrocytes (data not shown). This behaviour would not be considered important in the membrane-disrupting mechanism of C_{12}-HSL cytotoxicity. In contrast, the behaviour of erythrocytes treated with C_{12}-AR shown in Fig. 3 can be estimated as membrane destabilization. The differences between the sigmoid osmotic stability curves and values of the half-transition point of the hypotonic hemolysis (X_{50}) were significant for cells promoted by C_{12}-AR at concentrations above 5×10^{-5} M. The control X_{50} value was 0.46 ±0.03 g/dl NaCl, and when erythrocytes were pre-incubated with 5×10^{-5} M of C_{12}-AR this increased to 0.54 ±0.04 g/dl NaCl ($p \leq 0.001$); for 10^{-4} M C_{12}-AR-treated cells, the value increased to 0.78 ±0.04 g/dl NaCl ($p \leq 0.01$). This suggests that C_{12}-AR induced higher membrane permeability and affected the membrane stability, thus, operating in a similar fashion as other long-chain resorcinolic lipids [19, 20].

These data suggest that a significant part of C_{12}-AR, but not the C_{12}-HSL cytotoxic effect, is related to an interaction with membranes, and this interaction can lead to fast cell lysis (necrosis) without an induction of apoptosis.

Discussion

Cell death caused by small molecules originating from microbes (SMOMs) is one of the most extreme develop-

ments in the field of inter-kingdom relationships [1, 2]. We have made two conclusions by studying the cytotoxic mechanisms of two structurally close SMOMs: *N*-dodecanoyl-L-homoserine lactone and 5-*N*-dodecyl resorcinol. First, both compounds in micromolar concentrations showed significant dose-dependent cytotoxicity in granulocytes but not in monocytes. Second, these molecules use different mechanisms to impinge on the cell death pathway.

The present data demonstrate that C_{12}-HSL specifically induces apoptosis in granulocytes and monocytes. Recently, the pro-apoptotic activity has been reported for another HSL, a 3-oxo-*N*-dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) originating from *Pseudomonas aeruginosa* [14]. However, any changes in cell viability caused by other HSLs were still not observed. Our data for the first time demonstrate the specific ability, distinct from 3-oxo-C_{12}-HSL homoserine lactone, of C_{12}-HSL to induce cell death by apoptosis. On the other hand, we confirm that HSLs' fine structure, in particular the sufficient length of side chains, is very important for the ability of these molecules to express cytotoxic activity and induce apoptosis.

In addition to the known toxic and regulatory C_{12}-HSL functions, such as alteration of human cell cycling and metabolism, activation of NF-κB in macrophages [6] or increasing the expression of TNF-α, interleukin-1β, and interleukin-8 [5], the present data testify to the expressed immunomodulatory activity of this molecule. First and most obvious, because apoptosis is a critical event for immunological and inflammatory processes, its dysregulation may greatly favour the survival of HSL-producing bacteria in their hosts.

Despite the effective induction of apoptosis previously observed after treatment with some resorcinolic lipids [15, 16], we have not observed this activity for C_{12}-AR. In contrast, this molecule induced fast cytolytic effects shown by the release of cytoplasmic lactate dehydrogenase, and presumably was defined by cellular membrane destabilisation. Therefore, the ability of resorcinolic lipids to incorporate into the phospholipid bilayer [16], to induce higher membrane permeability, and to induce cell lysis [19] proves to be true. In accordance with the previously shown ability of some ARs to regulate bacteriolytic enzyme activity [20], and to block antigen-antibody interactions [21], the data gathered strongly suggest the involvement of this molecule in the regulation of the immune response.

In summary, the present data demonstrate that C_{12}-HSL and C_{12}-AR can eliminate key defence cells, such as monocytes and especially granulocytes, which would otherwise participate in the destruction of pathogenic bacteria. These results reinforce the SMOMs bifunctionality concept that such bacterial molecules not only regulate bacterial-bacterial interactions but also break immune defences as a new and important mechanism of host evasion.

The authors declare no conflict of interests.

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