Time and Concentration Dependent Influence of Dirithromycin on Neutrophils Oxidative Burst

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Dirithromycin is a 14-membered macrolide antibiotic, well known to yield high intragranulocytic levels after several hour exposure. We chose therefore to investigate oxidative metabolism after prolonged incubation periods with neutrophils.

Neutrophil generation of reactive oxygen species, represented by superoxide anion, was assessed after fMLP or Staphylococcus aureus-induced activation of the respiratory burst. Cellular uptake of the drug was assessed concurrently, in order to attempt a correlation with time-dependent modifications of the cellular oxidative status.

For 1 hour exposure time, a pro-oxidant effect was reported for lower concentrations, achievable during therapeutic administration, whereas the highest ones promoted a potent anti-oxidant effect. After prolonged incubation times, the anti-oxidant effect alone was reported, with time-dependent modifications of IC_{50} values. These values could be correlated with intracellular accumulation of the drug. The anti-inflammatory activity reported here for high dirithromycin concentrations, could be nevertheless clinically relevant, since dirithromycin cellular uptake extends beyond 4 hours.

Macrolides are antibiotics widely used in treatment of some infections involving bacteria with intracellular development[1,2]. This characteristic could arise from their antibacterial spectra, and from their great ability to enter phagocytes[3~6]. High intracellular concentrations could be responsible for modifications of host defense mechanisms, especially those ensure by release of proteolytic enzymes and reactive oxygen species (ROS)[7,8].

Indeed, phagocytosis of microorganisms by polymorphonuclear neutrophils (PMNs), resulting from binding on cell surface receptors, is responsible for NADPH-oxidase activation[9,10]. The enzyme activated complex, promotes a process called respiratory burst, inducing ROS production into the phagosomes[11]. The first species generated, superoxide anion ($O_2^{\cdot -}$), possesses a weak bactericidal activity. However, enzymatic reactions change $O_2^{\cdot -}$ into others reactive species, H_2O_2, HOCl, and $OH^{\cdot}$ endowed with high reactivity on intraphagosomal components[9,12].

Some macrolides are well-known to promote this PMN oxidative metabolism, allowing a pro-oxidant effect responsible for enhanced bactericidal activity[8,13]. However, high reactive species production could result in harmful process, as chronic infection occurs[12]. In this case, ROS are released with bacterial compounds into the surrounding medium, inducing a damaging effect on extracellular matrix[14]. Thus, antimicrobial agents could be of interest in such infections by ensuring, besides their antibacterial activity, an anti-inflammatory activity via an antioxidant effect on PMN metabolism[15~18].

The purpose of this study was to evaluate in vitro interaction between a macrolide antibiotic, dirithromycin, and superoxide anion production. Two agonists of the respiratory burst were used, which are among the most physiological stimulating agents. A bacterial suspension of Staphylococcus aureus should allow to

achieve an *in vitro* model of bacterial infection, whereas the *N*-formyl-Methionyl-Leucyl-Phenylalanine (fMLP), a bacterial tripeptide, will ensure protein kinase C (PKC) activation[19,20].

However, since previous investigations about dirithromycin were performed never exceeding 1 hour[8,21,22], we chose to realize prolonged incubation periods (up to 4 hours). Indeed, dirithromycin is characterized by a lipophilic structure[23], inducing a quite high capacity to penetrate into PMNs[2], and by basic properties probably responsible for a time-dependent intralysosomal accumulation[24]. We could therefore attempt a correlation of the measure of dirithromycin intragranulocytic concentrations for different exposure periods and modifications of superoxide production.

Materials and Methods

PMNs Suspension

PMNs were collected from heparinized blood of healthy volunteers by centrifugation ($400 \times g$, 30 minutes) on a polysucrose gradient (Histopaque®, Sigma)[25]. After mononuclear cells and Histopaque® removal, PMNs were recovered by haemolysis of red blood cells in ammonium chloride solution (NH_4Cl 150 mM, $NaHCO_3$ 10 mM, pH 7.4). PMN suspension purity was 95% or more, and trypan blue exclusion dye-test showed at least 95% of viability.

Drugs and Chemical Reagents

Dirithromycin and [^{3}H] dirithromycin (16.7 Ci/mmol, 0.05 mg/ml solution in ethanol-water 7/3) were kindly provided by Lilly Research Laboratories (Indianapolis, USA). Dirithromycin was further diluted in phosphate buffer saline (Na_2HPO_4, 100 mM). Buffers and chemical reagents were purchased from Sigma Chemical Company (St Quentin Fallavier, France).

Bacteria

Staphylococcus aureus were provided by Armentières Hospital (Armentières, France). Strains were resuspended to a concentration corresponding to a Mac Farland standard of 3 (Apisystem, Biomérieux).

Dirithromycin Cellular Toxicity

Dirithromycin dilutions devoid of cellular toxicity were assessed by measurement of lactate dehydrogenase (LDH) activity, a cytosolic enzyme, in the supernatant of the PMN suspension[26]. 2×10^6 cells were incubated at 37°C for different incubation periods, in presence or absence of 50 μl of each dirithromycin dilution (0.1 to 500 μg/ml). After centrifugation ($1125 \times g$, 5 minutes), LDH activity in the supernatant was evaluated with LDH Optimized Lactate-Dehydrogenase EC 1.1.1.27 UV-Test, on a Kontron Uvikon 860 spectrophotometer at 340 nm. LDH activity in the supernatant was expressed as the percentage of total LDH activity in the cell suspension[27].

Superoxide anion ($O_2^{\cdot -}$) Production

1×10^6 PMNs were preincubated at 37°C, for various exposure times (1 hour, 2 hours and 4 hours), in presence or absence of 100 μl of each dirithromycin dilution (0.1 to 400 μg/ml). $O_2^{\cdot -}$ generation was evaluated by superoxide dismutase-inhibitable reduction of cytochrome C^{28}. Dirithromycin activity on oxidative metabolism was expressed as percentage of inhibition or activation, of $O_2^{\cdot -}$ release in dirithromycin-free system[29].

Intracellular Accumulation of Dirithromycin

Triplicate samples containing 2.5×10^6 PMNs, dirithromycin (100 μg/ml) and [^{3}H] dirithromycin (5 μg/ml) were incubated at 37°C[24]. After different incubation periods, (1 hour, 2 hours and 4 hours), 100 μl aliquots were used to quantitated dirithromycin entry into PMNs. After 5 minutes centrifugation, 25 μl of supernatant or total cell pellet were introduced in 5 ml scintillation liquid (Ready Protein + ® - Beckman). Radioactivity was assessed in a Beckman L1800 liquid scintillation system.

PMN-associated and extracellular dirithromycin concentrations were calculated from PMNs intracellular volume ($2 \mu l/10^7$ cells)[6].

Statistical Analysis of Data

Data were expressed as mean values ± standard deviation (SD). Statistical analysis was performed by non-parametric Wilcoxon test, and statistical significance was defined as $p < 0.05$.

Results

Dirithromycin Cellular Toxicity

Dirithromycin concentrations ranging from 0.1 to 500 μg/ml were chosen in order to assess dirithromycin toxicity threshold.

Significant LDH release in the extracellular medium (Fig. 1), in comparison with the drug-free system, was reported for concentrations from 500, 400 and 300 μg/ml

Fig. 1. Dirithromycin impact on PMNs viability, assessed by LDH activity.

■ 1 hour incubation time, ● 2 hours incubation time, ▲ 4 hours incubation time.
$n = 6$, mean $\pm$ SD, $*p < 0.05$.

Dirithromycin (μg/ml)
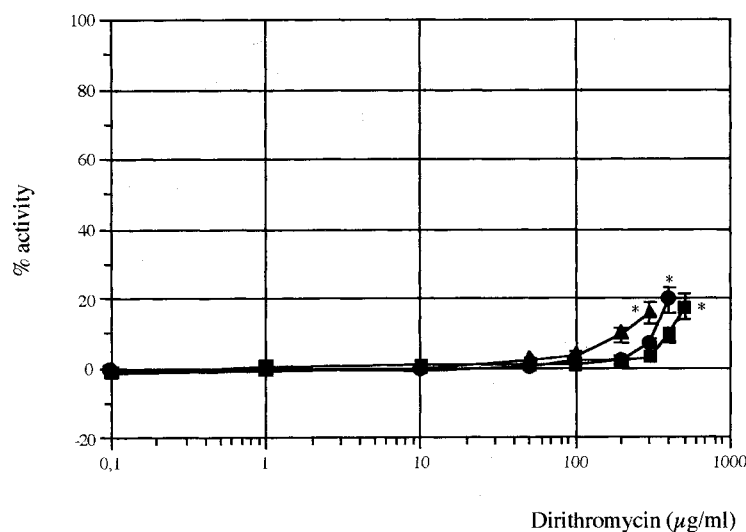
Fig. 2. Dirithromycin interaction with O_2^- generation by fMLP-induced human PMNs.

■ 1 hour incubation time, ● 2 hours incubation time, ▲ 4 hours incubation time.
$n = 6$, mean $\pm$ SD, $*p < 0.05$.

Dirithromycin (μg/ml)
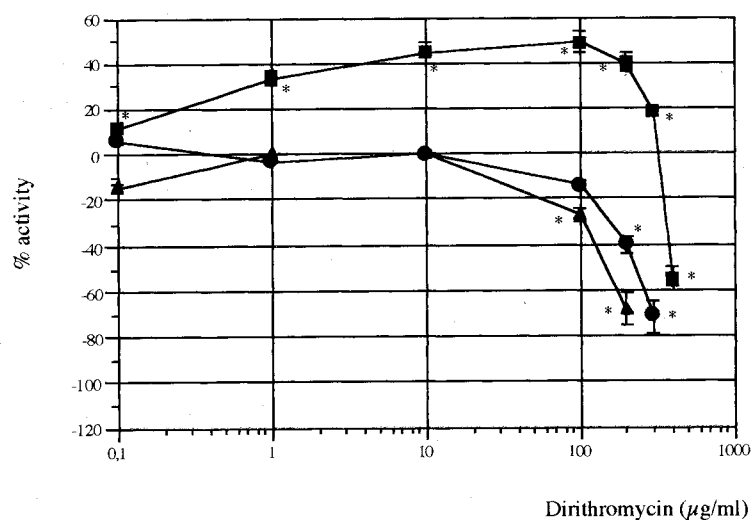
and above for 1 hour, 2 hours and 4 hours incubation times respectively.

Therefore, dilutions range between 0.1 and 400 μg/ml for 1 hour incubation, 0.1 and 300 μg/ml for 2 hours, and 0.1 and 200 μg/ml for 4 hours exposure times were used in order to achieve dirithromycin interaction with human neutrophils.

Superoxide Anion Production

As shown in figures 2 and 3, neutrophil stimulation by fMLP or *S. aureus* produced a significant pro-oxidant effect after short exposure time (1 hour) to low concentrations, including therapeutic ones: from 0.1 to 300 μg/ml, and 0.1 to 100 μg/ml for fMLP and *S. aureus* respectively. However, fMLP-activated neutrophils were the most sensitive to this effect (+52% for fMLP, and

Fig. 3. Dirithromycin interaction with O_2^- generation by *S. aureus*-induced human PMNs.

■ 1 hour incubation time, ● 2 hours incubation time, ▲ 4 hours incubation time.
$n = 6$, mean ± SD, * $p < 0.05$.

Dirithromycin (μg/ml)
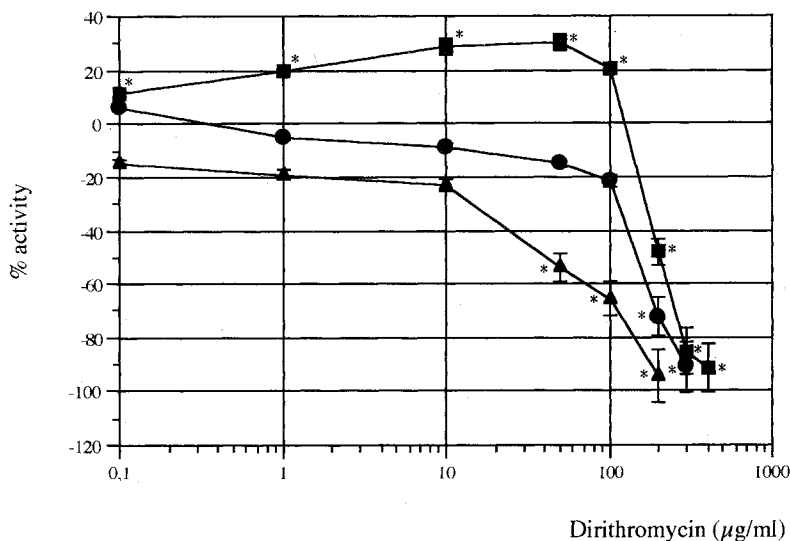
+28% for *S. aureus*). As extracellular concentrations increased, a marked antioxidant effect was observed, with IC_{50} values reaching 400 μg/ml and 194 μg/ml for fMLP and *S. aureus* respectively.

Increase of exposure time lead to a significant dose-related antioxidant effect reaching −71.4% for fMLP and −91% for *S. aureus*, for 2 hours exposure time. Moreover, this inhibitory effect was time-dependent, since IC_{50} values were 194 μg/ml, 109 μg/ml and 18 μg/ml for 1 hour, 2 hours, and 4 hours incubation periods with *S. aureus*-induced neutrophils. By using fMLP as stimulating agent, we reported a nearly similar effect with higher IC_{50} values: 400 μg/ml, 218 μg/ml and 152 μg/ml for the same incubation periods.

Intracellular Accumulation of Dirithromycin

The amount of cell-associated drug was determined in terms of the intracellular to extracellular ratio (C/E). Dirithromycin was massively accumulated by neutrophils in a time-dependent manner: C/E values were 6.29 +/− 1.15, 9.28 +/− 1.11 and 16.31 +/− 1.18 after 1 hour, 2 hours and 4 hours incubation times ($n = 4$).

Discussion

Dirithromycin is a 14-membered macrolide antibiotic, providing an improvement in pharmacokinetic[1]. Particularly, dirithromycin achieves high intratissular levels and a massive phagocytic uptake[24], responsible for a

time-dependent intracellular accumulation process, extending up to 24 hours. Despite of these characteristics, the large number of studies concerning dirithromycin impact on neutrophil oxidative metabolism, never exceeded 1 hour exposure time[8,19,22].

The aim of our study was to investigate the effect of dirithromycin on superoxide generation by human neutrophils, after prolonged incubation periods. This model should allow to attempt a correlation between time-dependent modifications of ROS production, and dirithromycin intracellular concentrations, measured concurrently.

The validity of these experiments was firstly demonstrated by assessment of dirithromycin molecular toxicity. Dirithromycin concentration range devoid of significant LDH extracellular release, was then used to perform a cell-free test, which did not show any dirithromycin scavenger activity[30]. These results concur with data provided by several authors[8,22]. The time-dependent modifications, which were observed here, seem therefore to stem from a cellular mechanism. Although, this one remains unclear, it seems possible to exclude a direct interaction with a specific cellular receptor, since results were nearly similar for the two different stimulating agents[8].

As low dirithromycin concentrations, including therapeutic ones were used, a pro-oxidant effect was observed. According to MOUTARD *et al.* (1996)[30], this one did not occur when Phorbol-Myristate-Acetate

(PMA) was used as stimulating agent. These data support the hypothesis that phospholipase C activation could be implied. After prolonged incubation times, no such pro-oxidant effect was reported. This is in keeping with observations made by JOONE et al. (1992)[22] with respect to oxidative burst stimulation by fMLP. Indeed, these authors explained that a prolonged preincubation could lead to a time-dependent decrease of fMLP-superoxide generating capacity.

This pro-oxidant effect obtained for lowest drug dilutions was not reported elsewhere. This discrepancy could be related to use of higher agonist concentrations which could create an higher responsiveness state.

On the contrary, the dirithromycin-induced depression of oxidative metabolism has been published yet[8,21,22], even if lower drug concentrations led to the same anti-oxidant effect. Two explanations could be raised i) most of experiments used the lucigenin-enhanced chemiluminescence (LECL) method, ii) employment by these authors of lower fMLP concentration, leading to a decrease of dirithromycin IC_{50} values.

With regard to the cellular mechanism underlying the antioxidant effect, several hypothesis have been formulated. Previous results, showing a potent PMA-induced antioxidant effect, could lead to different hypothesis[8,30]: either a single target of the transductional pathway is involved, which is located downstream to PKC[8,21], or most probably several impacts are concerned. So, according to HAND et al. (1993)[21], antibiotics which are characterized by basic properties and a marked intracellular accumulation, altered oxidative metabolism by PLD pathway inhibition. Dirithromycin was also reported to interact either with PKC, or with NADPH oxidase enzymatic complex[8,22,31]. Indeed, a recent study showed that macrolides anti-oxidant activity could be abolished by membrane destabilizing agents[32]. The dirithromycin-induced anti-inflammatory effect could therefore be due to a membrane stabilizing mechanism. HAND et al. (1990)[31] also support the hypothesis of an inhibitory effect directed on NADPH oxidase assembly. Indeed the translocation of the cytochrome b, being part of NADPH oxidase complex, could be depressed by intralysosomal accumulation of a weakly basic antimicrobial agent[33,34].

In the case of bacterial-induced model, IC_{50} values were lower, and time-dependent accumulation process was more efficient. A possible explanation was provided by PASCUAL et al. (1995)[35], who observed an increase of azithromycin C/E values, when S. aureus was used as stimulating agent, leading to higher intracellular concentrations. This mechanism could be imply for dirithromycin too, since intracellular accumulation process was similar[24,36].

All experiments published so far about dirithromycin phagocytic uptake established a lack of apparent saturation[21,22,24]. Thus, C/E values increased as a function of time up to 3 hours, whichever the extracellular concentration used. In contrast, these various studies led to quite heterogeneous results concerning both cellular uptake intensity and kinetic. For instance, MTAIRAG et al. (1994)[24] obtained an increase of C/E values from 30 to 47 between 1 and 2 hours incubation time, whereas data supplied by HAND et al. (1993)[21] were 19 and 34.5 for similar exposures. The former results are rather consistent with kinetic uptake of the present study. On the contrary, the latter showed lower values which are closer from our findings. Explanation for such differences was supplied by LAUFEN et al. (1990)[36], who evidenced an interindividual variance in intragranulocytic accumulation of azithromycin, from three different blood samples. Moreover, extracellular concentration (100 μg/ml) was chosed according to IC_{50} values firstly measured. However, since these one remains higher than in previous reports, and could result in weaker C/E values. All C/E values measurements never exceeded 4 hours, and correlation with IC_{50} decrease was therefore restricted to our own results. By using fMLP as stimulating agent, superoxide anion production was almost identical for similar intracellular concentrations, whatever the incubation time.

In conclusion, dirithromycin pharmacokinetic led us to realize prolonged incubation times. A time-dependent anti-oxidant effect appeared, which was attributed to an increase of intracellular drug levels. This leads one to suppose that even if IC_{50} values are quite higher than peak plasma concentration (C_{max} with 100 mg IV administration reached 2.37 μg/ml[23]), the potent intracellular accumulation process provides intraphagocytic concentrations likely to induce anti-oxidant effect. So, during therapeutic administration, the prolonged exposure to dirithromycin may probably provide a beneficial effect, by control of ROS deleterious effects, without impairement of antimicrobial functions.

Such clinical implication was recently suggested in view of macrolides efficacy on diffuse panbronchiolitis[37]. All data suggested that efficacy depended on mechanisms other than bacteriostatic activity, particularly decrease of oxidant production and immunosuppression.

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