Interaction of the New Ketolide ABT-773 (Cethromycin) with Human Polymorphonuclear Neutrophils and the Phagocytic Cell Line PLB-985 In Vitro

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A classical velocity centrifugation technique was used to study the in vitro uptake of the new ketolide ABT-773 by human polymorphonuclear neutrophils (PMNs) and a myelomonoblastic cell line, PLB-985, which can be differentiated into PMNs under certain culture conditions, compared to that of HMR 3004. ABT-773 was rapidly taken up by PMNs (cellular concentration to extracellular concentration ratio [C/E], about 34 at 30 s and up to 207 at 5 min), and uptake plateaued from 30 to 180 min (C/E, about 300). ABT-773 was accumulated significantly better than HMR 3004 from 5 to 180 min. Nondifferentiated PLB-985 cells (ND-PLB) accumulated significantly less ABT-773 and HMR 3004 than PMNs and PLB-985 cells differentiated into PMNs (D-PLB). Whatever the cell type and in contrast to the results obtained with HMR 3004, ABT-773 was mainly located in the cytosol (about 75%) and was rapidly released from loaded cells (about 40% at 5 min), followed by a plateau, likely owing to avid reuptake. Verapamil and H89, an inhibitor of protein kinase A, increased drug efflux. Uptake was sensitive to external pH, and the activation energy was moderate (about 50 kJ/mol). The existence of an active transport system on the PMN membrane was suggested by the following findings: concentration-dependent and saturable uptake (V_{max}, about 10 000 ng/2.5×10^6 PMNs/5 min; K_m, about 60 µg/ml) the inhibitory effects of PMN activators or inhibitors (phorbol myristate acetate, verapamil, Ni^{2+}) and the significantly decreased levels of accumulation by killed cells and cells treated at low temperatures. In addition, various macrolides impaired ABT-773 uptake, contrary to the findings for the quinolone levofloxacin. ND- and D-PLB also presented saturation kinetics that defined an active transport system (V_{max} and K_m values were similar to those obtained with PMNs), but the activation pathway of the carrier system did not seem to be fully functional in ND-PLB. As has been observed with other erythromycin A derivatives, ABT-773 impaired oxidant production by phagocytes in a time- and concentration-dependent manner. These data extend our previous results on the existence of an active transport system common to all macrolides and ketolides, at least in PMNs.

Ketolides are a new class of semisynthetic erythromycin A derivatives that are particularly designed to combat respiratory tract pathogens that have acquired resistance to macrolides (32). They are characterized by a 3-keto group instead of the usual α-L-cladinose moiety on the erythronolide A ring (6). Telithromycin (HMR 3647) is the first member of this new class to be approved for clinical use. Ketolides possess a broad antibacterial spectrum similar to that of erythromycin A, with additional activity against inducible macrolide-lincosamide-streptogramin B-resistant pathogens (4, 5). Activity against intracellular pathogens is the hallmark of macrolides and ketolides, but cellular uptake is required for these drugs to exhibit their activities (13, 14). Contrary to macrolides, for which an extended literature on cellular uptake is available, few data are available on the uptake of ketolides (2, 3, 20, 29–31). The uptake of the ketolides HMR 3004 (formerly RU 64004) (30) and HMR 3647 (telithromycin) (31) and the fluoroketolides HMR 3562 and HMR 3787 (2) have been analyzed previously.

ABT-773 (cethromycin), a new ketolide developed by Abbott, possesses good activity against gram-positive organisms, some gram-negative organisms, and intracellular bacteria (8, 11, 18). ABT-773 is active in vitro (9, 19, 24, 27) and in vivo (12, 21) against penicillin-resistant and inducibly erythromycin-resistant staphylococci, streptococci, and pneumococci. No data are yet available, however, on its uptake by phagocytes. In the study described here we investigated the interactions of ABT-773 (cethromycin) with human polymorphonuclear neutrophils (PMNs) and a phagocytic cell line (PLB-985) that can be differentiated into PMNs (25). HMR 3004 was used as a comparator drug. In addition, because all erythromycin A derivatives impair oxidant production by phagocytes (1, 29, 31), we also explored the effect of ABT-773 on this phagocyte function.

MATERIALS AND METHODS

Antibacterial agents. ABT-773 (cethromycin) and [^{14}C]ABT-773 (39.45 mCi/mmol in ethanol; radiochemical purity, >99.5%, as determined by high-pressure liquid chromatography) were provided by Abbott. Labeled HMR 3004 ([^{3}H]HMR 3004, 25.2 Ci/mmol) and unlabeled HMR 3004 were obtained from Aventis, Romainville, France. The standard solutions were prepared extemporaneously by adding 2.5 µl of the radiolabeled drug to 25 µl of the unlabeled solution (1,000 µg/ml) in Hanks balanced salt solution (HBSS; Sigma, St. Louis, Mo.) and adding that mixture to 222.5 µl of HBSS. Stock solutions were further diluted to reach the desired concentrations. Unlabeled roxithromycin, azithromycin, and levofloxacin were provided by Aventis.

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Culture medium and other materials. RPMI 1640 culture medium and fetal calf serum (FCS) were from Bio-Whittaker (Walkersville, Md.); penicillin and streptomycin were from Life Technologies (San Diego, Calif.); *N,N*-dimethyl-formamide (DMF) was from Merck (Darmstadt, Germany); nutridoma-SP was from Boehringer Mannheim (Basel, Switzerland); and phorbol-1,2-myristate-1,3-acetate (PMA), formylmethionyl-leucyl-phenylalanine, luminol, zymosan, and cytochrome *c* were from Sigma.

Human PMNs. PMNs were obtained from the venous blood of healthy volunteers by Ficoll-Paque centrifugation, followed by sedimentation with 2% dextran and osmotic lysis of residual erythrocytes. The viability and purity of the PMN preparation, as assessed by Trypan blue exclusion, were greater than 96%.

Culture and induction of PLB-985 cell differentiation. The human myeloid leukemia cell line PLB-985 (a generous gift from T. A. Rado, Birmingham, Ala.) was cultured in RPMI 1640–glutamine medium supplemented with 10% FCS, 50 U of penicillin per ml, and 50 μg of streptomycin per ml at 37°C in a humidified atmosphere of 5% CO_2 in air (25). Cell cultures were passaged two or three times a week to maintain a cell density between 2×10^5 and 1×10^6 cells/ml. For granulocyte differentiation, exponentially growing cells at a starting density of 2×10^5 cells/ml were cultured in RPMI 1640 medium supplemented with 0.5% DMF, 1% nutridoma-SP, and 0.5% FCS. The medium was changed once on day 3. By day 6, at least 95% of the cells had undergone granulocytic differentiation, on the basis of morphological and functional analyses. For the experiments reported here, nondifferentiated PLB-985 cells (ND-PLB) and PLB-985 cells differentiated for 6 days (D-PLB) were washed three times in HBSS without antibiotics and adjusted to the desired concentrations in fresh HBSS.

Measurement of cell volume. Cell volume was evaluated by two techniques: first, the total protein content of 2.5×10^6 cells was measured by spectrophotometry by a Bio-Rad protein assay, and the overall cell volume was calculated as described by Carlier et al. (7), in which 1 mg of protein corresponds to a cell volume of 5 μl. Second, the intracellular water space was measured by using [^{3}H]-labeled water (10 mCi/ml; Centre National de l'Énergie Atomique, Saclay, France); [^{14}C]polyethylene glycol (PEG; 0.8 mCi/mg; Dupont NEN Research Products, Boston, Mass.) was used as the extracellular marker, 2.5×10^6 cells were incubated with these radiolabeled compounds at 37°C for 5 min before centrifugation through a water-impermeable silicone-paraffin oil cushion, and a Beckman scintillation counter was then used to determine the counts in the cell pellet. The total water content was corrected for the trapped extracellular water, i.e., the PEG space, to obtain the intracellular water space. In agreement with published data (18), we had previously determined the water compartment of 2.5×10^6 PMNs to be about 0.6 μl. In the present study we confirmed this value in three experiments (mean ± 1 standard deviation [SD], 0.6 ± 0.07 μl). For 2.5×10^6 PLB-985 cells we determined the water compartment to be about 1.2 ± 0.17 μl (ND-PLB) (three experiments) and 0.98 ± 0.03 μl (D-PLB) (five experiments). The protein-based assay (for determination of the overall volume) gave approximately twice these values (PMNs, 1.22 μl [one experiment]; ND-PLB, 2.1 ± 0.26 μl [four experiments]; D-PLB, 2.2 μl [one experiment]). To calculate intracellular concentrations, values of 0.6 μl (PMNs), 1.2 μl (ND-PLB), and 1 μl (D-PLB) for 2.5×10^6 cells were used. We verified that the various experimental conditions used here (temperature, pH, presence of inhibitors) did not significantly modify these values.

Uptake kinetics. A classical radiometric assay was used to determine uptake kinetics (22, 23). Briefly, 2.5×10^6 cells were incubated with the radiolabeled drugs at 37°C and were then centrifuged at $12,000 \times g$ for 3 min at 22°C through a water-impermeable silicone-paraffin oil (86 and 14% [vol/vol], respectively) barrier. The pellet was solubilized in Hionic fluor (Packard), and the cell-associated radioactivity was quantified by liquid scintillation counting (LS-6000; Beckman). Standard dilution curves were used to determine the amounts of cell-associated drug. The results were expressed as nanograms per 2.5×10^6 cells. The concentration of each of the ketolides used in the assays was 2.5 μg/ml unless otherwise indicated. The volumes of the intracellular compartments determined as described above were used to calculate the cellular concentration/extracellular concentration ratio (C/E) in PMNs.

Intracellular location. A total of 2.5×10^6 cells were loaded with the drugs at 10 μg/ml (30 min at 37°C) and centrifuged through the oil cushion. The cell pellet was sonicated in the presence of 0.5% Triton X-100 or 0.73 M sucrose to protect the granules (17, 23, 24). After centrifugation, the amounts of lysozyme (a granule marker) and radiolabeled drugs in the pellet and the supernatant were determined as described previously (23).

Ketolide efflux. Aliquots of ketolide-loaded cells (30 min at 37°C, 10 μg/ml) were centrifuged and then placed in drug-free medium. At various times they were again centrifuged through an oil cushion, and the radioactivities in the pellet and the supernatant were determined. Efflux was quantified as the percentage of drug released in the supernatant relative to the sum of the amounts

of drug in the pellet and the supernatant. This sum did not differ significantly from the total amount of cell-associated drug measured in a control aliquot of ketolide-loaded cells.

Characteristics of ketolide uptake. Various experimental conditions were used to study the mechanism of uptake, as follows: cell viability (pretreatment of PMNs with 10% formaldehyde, followed by two washes in HBSS); pH, 7 to 9; temperature, 0, 20, 37, and 40°C; extracellular concentrations, 2.5 to 50 μg/ml; and pretreatment for 15 min with various metabolic inhibitors (NaF, KCN, or 2,4- dinitrophenol at 1 mM each) or with various activators or inhibitors of PMN functions which have been reported to interfere with macrolide uptake (17, 23, 24), namely, Ni^{2+}, a blocker of the Na^+-Ca^{2+} exchanger (0.625 to 5 mM); PMA, a protein kinase C (PKC) activator (100 and 10 ng/ml); H89, a protein kinase A (PKA) inhibitor (50 μM); and verapamil, a Ca^{2+} channel blocker (62.5 to 250 μM). We also studied the inhibitory effects of various macrolides and ketolides, and the quinolone levofloxacin (100 μg/ml) was used as a control of passive accumulation (28). Concentration dependence experiments were performed in the presence of unlabeled drugs.

PMN viability. PMN viability was assessed by measuring the amount of lactic dehydrogenase released by PMNs incubated in the presence of the drugs. Under the experimental conditions used here, none of the ketolides significantly impaired cell viability.

Oxidant production. Oxidant production was determined as follows. First, the level of superoxide anion production was measured by monitoring the level of superoxide dismutase-inhibitable cytochrome *c* reduction at 550 nm in a UVIKON 860 spectrophotometer (1). PMA (100 ng/ml) was used as stimulating agent. A total of 10^6 PMNs were incubated with buffer (control) or with ABT-773 solutions (0.1 to 100 mg/liter) at 37°C before the addition of cytochrome *c* and PMA. The level of cytochrome *c* reduction was recorded continuously for 400 s; the overall level of production over the entire period and the maximal rate of superoxide anion production (slope of the curve) were determined by using an extinction coefficient of 21,100 M^{-1} cm^{-1}.

Second, the overall level of oxidative burst was measured in terms of luminol-amplified chemiluminescence (LACL). Briefly, 10^6 PMNs were incubated with control buffer or the corresponding ABT-773 solutions for 30 or 60 min at 37°C in the dark; then, luminol (100 μM) in HBSS) and cells were stimulated with PMA (100 ng/ml) or opsonized zymosan (2.5 mg/ml); chemiluminescence was measured with a chemiluminometer (Autolumat LB953; Berthold, Bad-Wildbad, Germany) for 15 min at 37°C.

Statistical analysis. The results are expressed as the means ± 1 SD of *n* experiments conducted with PMNs from different volunteers or PLB-985 cells from different cultures. Analysis of variance, regression analysis, and Student's *t* test were used to determine statistical significance. All tests were performed with the Cricket software Statworks program, version 1.2 (1985).

RESULTS

Uptake kinetics. ABT-773 was rapidly taken up by PMNs and PLB-985 cells, with C/Es of about 34 (PMN), 26 (D-PLB), and 21 (ND-PLB) in the first 30 s; and uptake was progressive up to 30 min (Fig. 1B); uptake then plateaued in PMNs (C/E range, 318 ± 35.9 at 30 min [eight experiments] to 284 ± 96.0 at 180 min [five experiments]) ($P > 0.05$ versus the results at 30 min); in PLB-985 cells, the level of uptake increased up to 30 min, and then there was a decrease that was significant at 180 min for D-PLB ($P = 0.022$ versus the results at 30 min) and from 60 to 180 min for ND-PLB ($P = 0.027$, 0.014, and 0.020 for 30 min versus the results at 60, 120, and 180 min, respectively). The overall levels of accumulation (Fig. 1A) were similar for PMNs and D-PLB; however, owing to the greater cell volume for D-PLB, the capacity to accumulate the drug (C/E) in PMNs was superior to that in D-PLB (Fig. 1A). ND-PLB displayed a significantly inferior level of accumulation compared to those of PMNs and D-PLB (Fig. 1). As reported previously (30), HMR 3004 was also rapidly accumulated by PMNs to a degree significantly inferior to that of ABT-773 from 5 to 120 min ($P = 0.016$ at 5 min, $P \leq 0.001$ at 30 and 60 min, and $P = 0.008$ at 120 min). The level of accumulation of
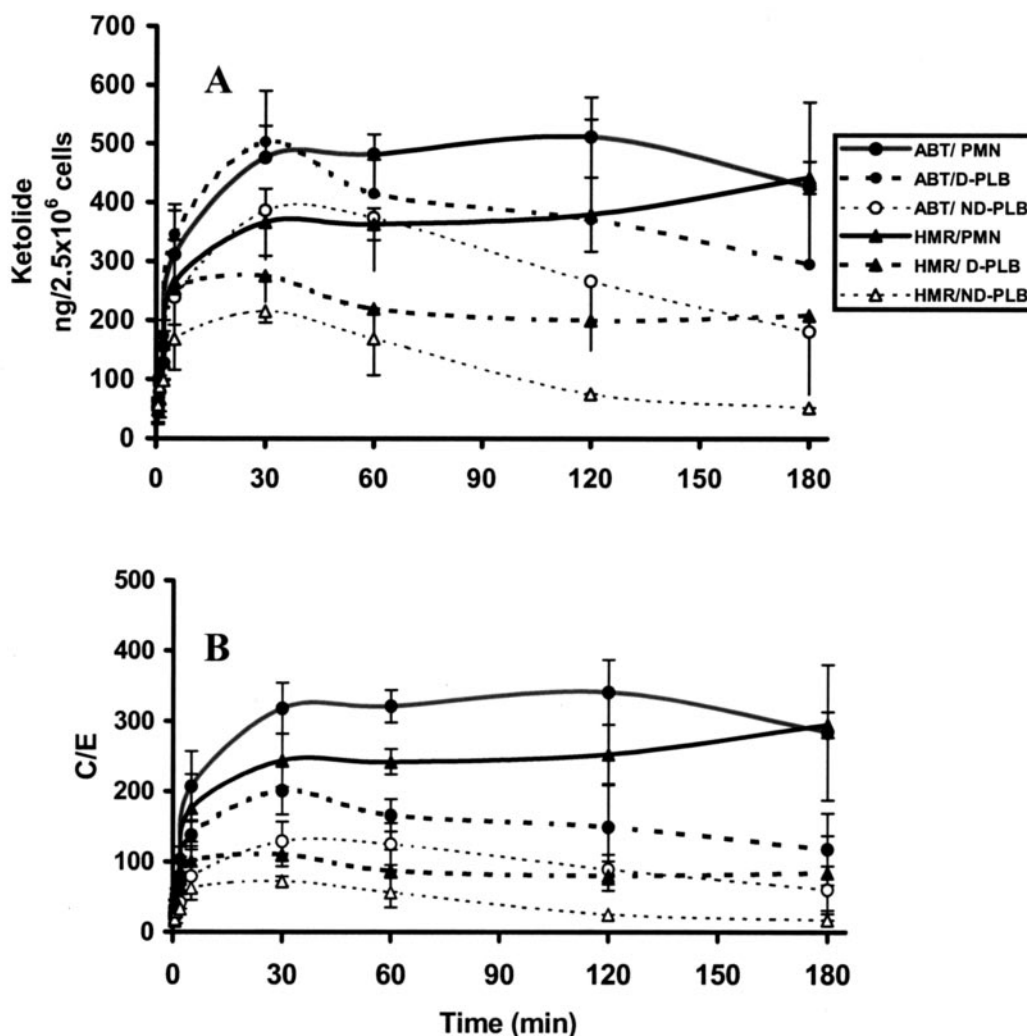
FIG. 1. Uptake kinetics of ABT-773 (ABT) and HMR 3004 (HMR) by evaluation of comparative accumulation in PMNs, ND-PLB, and D-PLB. (A) Overall accumulation (nanograms per 2.5×10^6 cells); (B) C/E (means $\pm$ 1 SD) in 5 to 21 experiments with PMNs from different individuals, 3 to 12 experiments with D-PLB, and 3 to 13 experiments with ND-PLB.

HMR 3004 was also less than that of ABT-773 in D-PLB from 5 to 60 min and in ND-PLB at 5 and 30 min.

Cellular location. The granule enzyme marker lysozyme was used to identify the granular compartment of PMNs (about $91\% \pm 6.5\%$ of the total enzyme); the Triton X-100-insoluble fraction (membranes plus unbroken granules) contained about $14\% \pm 3.3\%$ of total lysozyme. The proportions of the cytosolic marker lactic dehydrogenase were less than 6% in the granule fraction and about 4% in the Triton X-100-insoluble fraction. ABT-773 was mainly located (about $75\% \pm 2.1\%$) in the cytosolic compartment of PMNs; less than 5% ($4\% \pm 2.1\%$) was associated with the membrane (Triton X-100-insoluble) fraction (four experiments). In differentiated PLB-985 cells, $22\% \pm 6.4\%$ of the ABT-773 was associated with the granular compartment and $2\% \pm 1.5\%$ was associated with the membranes (three experiments). ABT-733 was observed in similar locations in ND-PLB: $24\% \pm 18.7\%$ was in the granular compartment, and $2.3\% \pm 0.4\%$ was associated with the membranes (two experiments). As reported previously, HMR 3004

accumulated better in the granular compartment of PMNs ($65\% \pm 13.3\%$), and about $4\% \pm 3.2\%$ was associated with the membranes. The percentage associated with the granules in D-PLB and ND-PLB was less than that associated with the granules in PMNs ($29\% \pm 4.2\%$ and 32%, respectively), but the levels of membrane binding were similar for D-PLB (two experiments) and ND-PLB (one experiment) ($1.3\% \pm 1.0\%$ and 2%, respectively).

Efflux kinetics. ABT-773 was rapidly released from loaded cells (Fig. 2). The rate of efflux was maximal in the first 5 min (about 9%/min), and then there was a plateau owing to the equilibrium between entry and efflux, as suggested by the following. When PMNs loaded with ABT-773 were incubated in drug-free medium for 30 min, centrifuged, and again placed in fresh drug-free medium, the ketolide egressed from the cells; and less than 10% remained associated with the cell pellet within 30 min of incubation. The release of ABT-773 was significantly greater than that of HMR 3004 (release of 6%/min in the first 5 min, followed by a plateau, with about 60 to
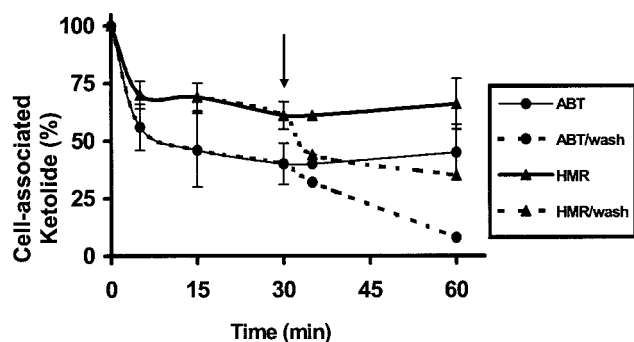
FIG. 2. Ketolide efflux. PMNs loaded with the ketolides ABT-773 (ABT) and HMR 3004 (HMR) (10 mg/liter) were isolated and placed in drug-free medium (see Materials and Methods). The results are expressed as the amount of pellet-associated ketolides as a percentage of the total amount after various times in drug-free medium. The arrow indicates when PMNs were again centrifuged and incubated in fresh drug-free medium (wash). The values are the means ± 1 SD of seven experiments for ABT-773 and four experiments for HMR 3004. P was <0.05 for ABT-773 versus HMR 3004 at all times.

66% remaining in the cell pellet at 30 and 60 min). When PMNs loaded with HMR 3004 were incubated in drug-free medium for 30 min, centrifuged, and again placed in fresh drug-free medium, the ketolide egressed from the cells and about 35% remained associated with the cell pellet within 30 min of incubation (Fig. 2). ABT-773 and HMR 3004 were also quickly released from loaded PLB-985 cells: the ABT-773 efflux rates were 11 and 8%/min for D-PLB and ND-PLB, respectively; and the HMR 3004 efflux rates were 10 and 10.6%/min, respectively, during the first 5 min, followed by a plateau for D-PLB. In ND-PLB cells, however, efflux continued over 30 min (cell-associated ketolide levels were 34% ± 15.5% for ABT-773 [three experiments; $P < 0.05$ versus the results for 5 and 15 min] and 36% for HMR 3004 [one experiment]), likely because of the lower accumulation rates in these cells. As observed with other erythromycin A derivatives, ketolide efflux was increased in the presence of verapamil or H89 in PMNs and PLB-985 cells. The efflux rate was almost doubled in the first 5 min in the presence of either verapamil (125 μM) or H89 (50 μM); in PMNs, the effect of verapamil was prolonged, with less than 5% remaining associated with PMNs after 30 min of incubation, suggesting that verapamil both increases drug efflux and inhibits drug reuptake. By contrast, the effect of H89 was demonstrated only in the first 5 min, followed by a plateau, suggesting that this PKA inhibitor does not interfere with drug entry. For PLB-985 cells, verapamil and H89 resulted in more than 80% release in the first 5 min.

Analysis of the mechanism underlying cellular ketolide uptake. (i) Effects of temperature and cell viability. ABT-773 was poorly accumulated by PMNs at 0°C (Fig. 3). The maximal amount of cell-associated drug was obtained in the first 5 min (C/Es, about 17 at 5 min and about 12 at 180 min), without further changes. At 20°C there was a gradual accumulation (C/E range, about 39 [5 min] to 46 [60 min]). Uptake was more rapid at 40°C than at 37°C (C/E, 253,218 [$P < 0.05$]), but there were no significant differences at further incubation times. The activation energy (ΔG; in calories per mole) was calculated as described previously (14, 23, 24) after incubation of PMNs and
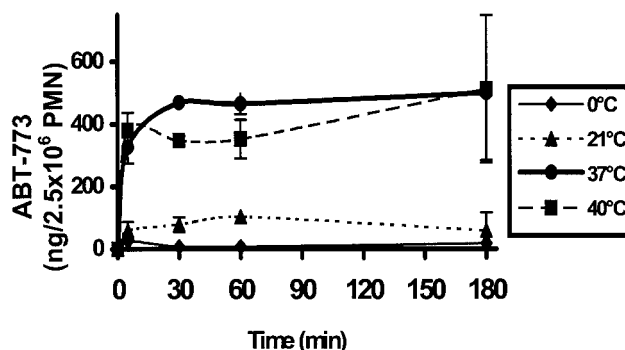

FIG. 3. Effect of temperature on cellular uptake of ABT-773 by PMNs. The results are expressed as nanograms per 2.5×10^6 PMNs and are the means of 3 to 12 experiments.

ketolides at 0, 20, 37, and 40°C for 5 min by using the Arrhenius equation: $\Delta G = -RT \ln K_{eq}$, where R is a constant (1.98 cal/mol), T is temperature (in degrees Kelvin), and K_{eq} is the C/E at 5 min. ΔG values for ABT-773 were 50 kJ/mol for PMNs, 40 kJ/mol for D-PLB, and 37 kJ/mol for ND-PLB. Viability was required for optimal uptake. When formaldehyde-killed cells were used, the level of ketolide uptake was very low. After 30 min of incubation, the level of passive association with PMNs and PLB-985 cells was about 30% (PMNs, 24% ± 12.4% [three experiments]; D-PLB, 35% ± 12.0% [three experiments]; ND-PLB, 43% ± 15.3% [five experiments]). Similar results were obtained with HMR 3004.

(ii) Effects of pH. ABT-773 displayed moderate susceptibility to a variation in the pH of the medium; the accumulation rate at 5 min was low at acidic pH. The mean values at pH 6.5 and 6.0 were as follows: for PMNs, 71 and 30 ng/2.5×10^6 cells/5 min, respectively (four experiments); for D-PLB, 60 and 20 ng/2.5×10^6 cells/5 min, respectively (two experiments); and for ND-PLB, 25 and 19 ng/2.5×10^6 cells/5 min, respectively (two experiments). The uptake of ABT-773 increased at pHs of 7 and higher (Fig. 4). We determined the pH dependence by regression analysis in PMNs as follows: $[C^n] = 144.3$ pH − 837.2 ($P < 0.001$; $r = 0.990$), where C^n is cell-associated ketolide (ng/2.5×10^6 PMN). Similar data were obtained for HMR 3004 by regression analysis: $[C^n] = 83.3$ pH − 406.12 ($P < 0.001$; $r = 0.693$).

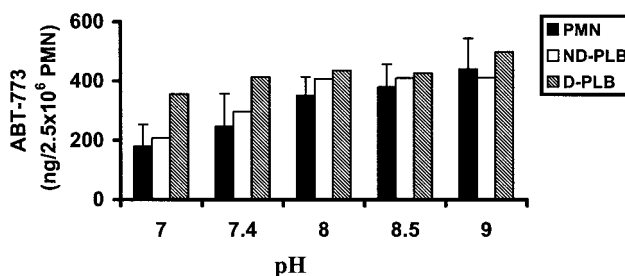FIG. 4. Effect of pH on cellular uptake of ABT-773 by PMNs, ND-PLB, and D-PLB. PMNs were incubated for 5 min. The results are the means ± 1 SD of five experiments for PMNs and the means of only two experiments for ND-PLB and D-PLB.
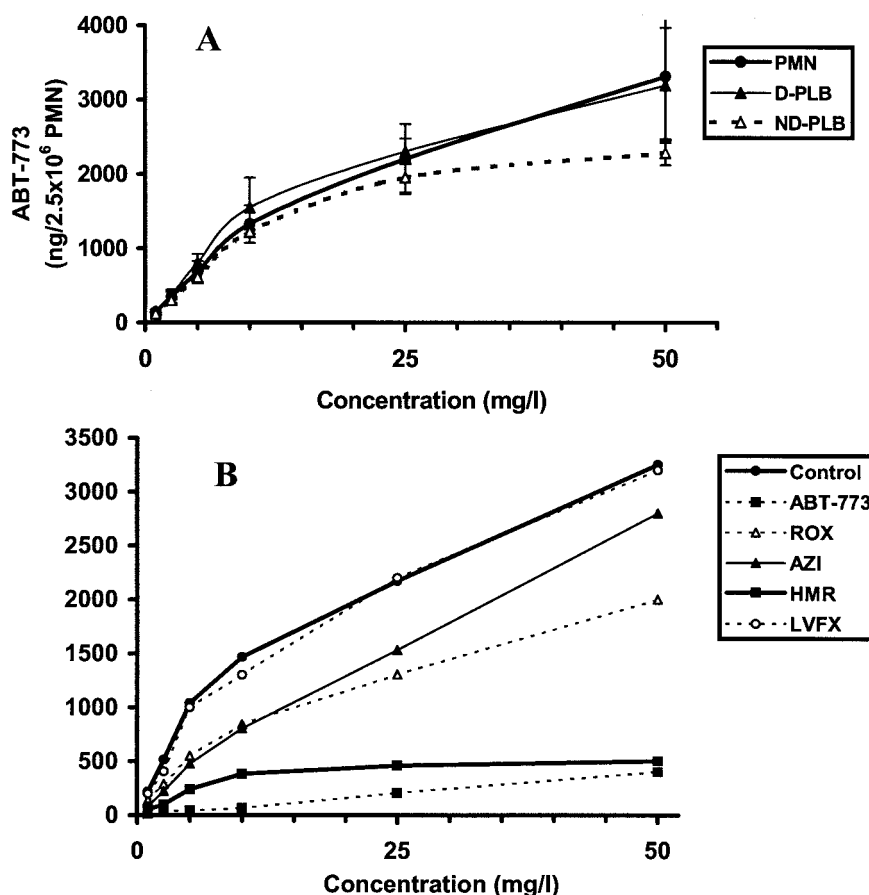
FIG. 5. Effects of the extracellular concentration on cellular uptake of ABT-773 by PMNs, ND-PLB, and D-PLB at 5 min. (A) The results are expressed as nanograms per 2.5×10^6 cells/5 min and are the means of five experiments for PMNs and three experiments for ND-PLB and D-PLB. (B) Inhibitory effects of macrolides and ketolides on ABT-773 uptake by PMNs. PMNs were incubated with HBSS (control) or unlabeled roxithromycin (ROX; 100 mg/liter), azithromycin (AZI; 100 mg/liter), ABT-773 (ABT; 100 mg/liter), HMR 3004 (HMR; 10 mg/liter), or levofloxacin (LVFX; 100 mg/liter) for 5 min; and then radiolabeled ABT-773 was added for 5 min and uptake was measured as described in the text. The results are the means of two to four experiments.

(iii) Effects of extracellular concentrations. Extracellular drug concentrations of 1 to 50 μg/ml were tested (Fig. 5A). Accumulation at 5 min displayed saturation kinetics characteristic of those for a carrier-mediated transport system. Mean V_{max} and K_m values were calculated from Lineweaver-Burk plots of three (PLB) to five (PMN) experiments, as follows: for PMNs, $V_{max} = 10,331 \pm 9,621$ ng/2.5×10^6 PMNs/5 min and $K_m = 72 \pm 66.6$ mg/liter ($P < 0.001$; $r = 0.999$); for D-PLB, $V_{max} = 9,215 \pm 3,604$ ng/2.5×10^6 D-PLB/5 min and $K_m = 59 \pm 17.0$ mg/liter ($r = 0.998$), and for ND-PLB, $V_{max} = 6,217 \pm 273$ ng/2.5×10^6 ND-PLB/5 min and $K_m = 49 \pm 10.0$ mg/liter ($r = 0.998$). In three experiments we confirmed previously published data for HMR 3004 and PMNs: $V_{max} = 3,052 \pm 2,983$ ng/2.5×10^6 PMNs/5 min and $K_m = 25 \pm 23.1$ mg/liter ($r = 0.995$) We also calculated the values of the parameters for D- and ND-PLB (two experiments), which were as follows: $V_{max} = 1,335 \pm 123.7$ ng/2.5×10^6 D-PLB/5 min and $K_m = 7.5 \pm 2.1$ mg/liter ($r = 0.995$) for D-PLB and $V_{max} = 2,056 \pm 896$ ng/2.5×10^6 ND-PLB/5 min and $K_m = 18 \pm 11.5$ mg/liter ($r = 0.996$) for ND-PLB. The carrier system seems to be present on nondifferentiated phagocytic cell lines, but its functionality de-

pends on the level of cell maturation. As observed previously (30), great variability in the parameters of macrolide uptake exists among PMNs from different donors.

We then analyzed whether a common carrier was involved in ketolide uptake. PMNs were first incubated for 5 min with ABT-773, roxithromycin, azithromycin, or levofloxacin (each at 100 mg/liter) or HMR 3004 (at 10 mg/liter) and were further incubated with the corresponding labeled drug at 1 to 50 μg/ml for 5 min; the levels of accumulation in the cells were then measured (Fig. 5B). The inhibitory concentrations chosen slightly exceed the K_m values for the various macrolides or ketolides obtained here and in previous reports (30, 31). For HMR 3004, the inhibitory concentration chosen was the lowest value obtained in the preliminary experiments given above. Because we observed in preliminary experiments that levofloxacin inhibited ABT-773 uptake due to its acidic pH in solution, we buffered it at pH 7.4 for the inhibition experiments.

The macrolides and ketolides, but not levofloxacin, inhibited ABT uptake (Fig. 5B). The kinetic parameters of ABT-773 were then calculated as described above and were as follows:

TABLE 1. Effects of competitive and metabolic inhibitors on
ABT-773 uptake

Inhibitor (concn [mM])[a]	% of control uptake (mean ± 1 SD)	
	PMN	ND-PLB
D-Glucose (1)	106 ± 19.4 (7)[b]	101 ± 47.7 (3)
L-Cys (1)	101 ± 25.1 (4)	102 (1)
L-Phe (1)	137 ± 49.5 (3)	98 (1)
L-Tyr (1)	143 ± 68.3 (3)	ND[c]
L-Met (1)	138 ± 70.7 (3)	ND
NaF (1)	98 ± 24.5 (6)	90 ± 16.0 (4)
KCN (1)		
pH 9	135 ± 5.6 (4)[d]	126 (1)
pH7.4	89 ± 34.4 (4)	98 (1)
NaN$_3$ (1)	75 ± 18.9 (4)	134 ± 15.6 (2)
2,4-DNP (0.1)	89 ± 23.1 (4)	92 ± 29.8 (3)

[a] Abbreviations: Cys, cysteine; Phe, phenylalanine; Tyr, tyrosine; Met, methionine; 2,4-DNP, 2,4-dinitrophenol.
[b] The number of experiments is given in parentheses.
[c] ND, not done.
[d] Significantly different (P < 0.001) from the results for the control (100%).

TABLE 2. Effects of PMN activators or inhibitors on
ABT-773 uptake

Inhibitor (concn)	% of control uptake (mean ± 1 SD)		
	PMNs	ND-PLB	D-PLB
PMA			
100 ng/ml	30 ± 8.4 (9)[a,b]	99 ± 8.2 (6)	70 ± 4.8 (4)[b]
10 ng/ml	36 ± 9.8 (5)[b]	ND[c]	74 ± 6.4 (3)[b]
VPL			
250 μM	25 ± 14.6 (7)[b]	41 ± 28.7 (3)	44 ± 3.5 (2)
125 μM	52 ± 7.2 (9)[b]	62 ± 33.1 (6)[b]	67 ± 4.7 (3)[b]
H89 (50)	71 ± 19.6 (3)	83 ± 26.5 (3)	92 ± 7.1 (2)
Ni^{2+}			
5 mM	29 ± 13.9 (6)	41 ± 11.4 (6)[b]	45 ± 7.8 (2)
2.5 mM	62 ± 27.2 (4)	55 ± 27.9 (4)[b]	53 (1)

[a] The number of experiments is given in parentheses.
[b] Significantly different (P < 0.001) from the results for the control (100%).
[c] ND, not done.

for the control, V_{max} = 4,717 and K_m = 19 (r = 0.999); for roxithromycin, V_{max} = 2,590 and K_m = 21 (r = 0.995); for azithromycin, V_{max} = 10,638 and K_m = 117 (r = 0.992); for HMR 3004, V_{max} = 739 and K_m = 16 (r = 0.917); and for ABT-773, V_{max} = 333 and K_m = 26 (r = 0.994). These data indicate that, except for azithromycin, which appears to be a competitive inhibitor, the other macrolides and ketolides are noncompetitive inhibitors (similar K_m values and strongly reduced V_{max} values).

Effects of inhibitors. None of the inhibitors of known transport systems (glucose, amino acids) on the PMN membrane or metabolic poisons interfered with ABT-773 uptake by PMNs or PLB-985 cells (Table 1). Only KCN significantly increased drug uptake, but this was due to its alkaline pH. It has been reported previously that PKC activation by PMA impairs roxithromycin uptake (29). Similar data have been obtained with all erythromycin A derivatives (M. T. Labro, unpublished data). By contrast, the PKA inhibitor H89 impairs roxithromycin and HMR 3004 uptake, whereas it increases azithromycin and HMR 3647 uptake. The Ca^{2+} channel inhibitor verapamil displays similar activating or inhibitory properties according to the macrolide or ketolide subgroup (22, 30, 31), whereas Ni^{2+},

which blocks the Na$^+$-Ca^{2+} exchanger on the PMN membrane, impairs macrolide uptake, whatever the subgroup (22, 30, 31). We tested the effects of various PMN activators and inhibitors on the uptake of ABT-773 by PMNs and PLB-985 cells (Table 2).

ABT-773 uptake by PMNs was strongly inhibited by the PKC activators PMA, verapamil, and nickel. The effect of PMA was also demonstrated for ABT-773 uptake by D-PLB (although to a lesser extent than in PMNs) but not by ND-PLB. Verapamil and nickel interfered with the uptake, whatever the cell type. The 50% inhibitory concentrations (IC$_{50}$; i.e., the concentrations that inhibited drug uptake by 50%) of nickel and verapamil were determined. They were as follows: for nickel, 3.5 mM for PMNs, 3.9 mM for D-PLB, and 3.8 mM ND-PLB; for verapamil, 149 μM for PMNs, 210 μM for D-PLB, and 192 μM for ND-PLB. Similar results were obtained with HMR 3004; and in particular, PMA strongly inhibited drug uptake by PMNs, but this effect was less important in D-PLB and there was no inhibition for ND-PLB.

Effects of ABT-773 on oxidant production by PMNs. As already observed with all erythromycin A derivatives, including the ketolides, ABT-773 impaired oxidant production by PMNs

TABLE 3. Effects of ABT-773 on oxidant production by PMA-stimulated PMNs

ABT-773 concn (mg/liter)	Effect of ABT-733 after incubation for[a]:			
	30 min		60 min	
	Overall production	Slope	Overall production	Slope
Control	5.6 ± 2.67 (7)[b]	8.4 ± 4.9 (7)	3.6 ± 1.75 (6)	5.3 ± 3.06 (6)
50	15 ± 17.0 (5)[c]	18 ± 22.7 (5)[c]	0.75 ± 1.5 (4)[c]	0.5 ± 1.0 (4)[c]
25	41 ± 29.4 (5)[c]	52 ± 30.2 (5)[d]	11 ± 19.6 (3)[c]	11 ± 18.5 (3)[c]
10	69 ± 13.2 (7)[d]	75 ± 18.9 (7)[e]	87 ± 22.4 (5)	85 ± 23.2 (5)
1	101 ± 27.5 (5)	84 ± 16 (5)	102 ± 29.8 (5)	115 ± 33.3 (5)
0.1	88 ± 16.8 (5)	85 ± 21.4 (5)	115 ± 28.9 (4)	122 ± 2.5 (4)

[a] The results are expressed as the mean ± 1 SD of reduced cytochrome c (nanomoles per 10^6 PMNs per minute) for the control and the percentage of the control response for the different concentrations of ABT-773. For technical details, see Materials and Methods.
[b] The number of experiments is given in parentheses.
[c] Significantly different (P < 0.001) from the results for the control.
[d] Significantly different (P = 0.001) from the results for the control.
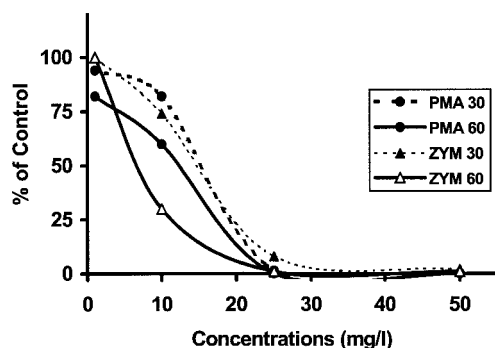[e] Significantly different (P = 0.012) from the results for the control.

FIG. 6. Effect of ABT-773 on PMNs as determined by LACL. PMNs were incubated for 30 or 60 min in the presence of buffer or ABT-773 (1 to 50 mg/liter) before stimulation with PMA (100 ng/ml) or opsonized zymosan (ZYM; 2.5 mg/ml). The results are expressed as the percentage of the control response and are the means of six to eight experiments. Control responses were as follows: with PMA stimulation for 30 min of incubation (PMA 30), $(14.5 \pm 7.53) \times 10^6$ cpm/1 $\times 10^6$ PMNs; with PMA stimulation for 60 min of incubation (PMA 60), $(17.5 \pm 8.38) \times 10^6$ cpm/1 $\times 10^6$ PMNs; with opsonized zymosan stimulation for 30 min of incubation (ZYM 30), $(1.8 \pm 0.91) \times 10^6$ cpm/1 $\times 10^6$ PMNs ($P = 0.01$ versus the results for PMA); with opsonized zymosan stimulation for 60 min of incubation (ZYM 60), $(1.8 \pm 0.92) \times 10^6$ cpm/1 $\times 10^6$ PMNs ($P = 0.012$ versus the results for PMA).

in a time- and concentration-dependent manner (Table 3 and Fig. 6). The first step of NADPH oxidase activation was explored by determination of the superoxide dismutase-inhibitable reduction of cytochrome c after PMA stimulation (Table 3). The IC$_{50}$s of ABT-773 which impaired the production of superoxide anion were calculated from logarithmic regression analysis as 14 mg/liter after 30 min of incubation and 10.7 mg/liter after 60 min of incubation ($P < 0.001$; $r = 0.704$ and 0.783, respectively). When the effect of ABT-773 on the oxidative metabolism of PMNs was assessed by a global technique (LACL), a similar inhibitory effect was observed, whatever stimulus was used, either PMA, which bypasses receptors and directly activates PKC, or opsonized zymosan, which activates phagocytic receptors. The IC$_{50}$s of PMA and zymosan were 8 and 14 mg/liter, respectively, after 30 min of incubation ($P < 0.001$; $r = 0.731$ and 0.790, respectively) and 4 and 9 mg/liter, respectively, after 60 min of incubation ($P < 0.001$; $r = 0.769$ and 0.911, respectively). These values are in the same range as those obtained by the cytochrome c assay.

DISCUSSION

The intracellular accumulation of macrolides is required for these drugs to be active against intracellular pathogens (13, 14) and, probably, for them to exhibit their potential anti-inflammatory activities (10, 15, 16). Among the recent areas of progress that has been made with this antibiotic family, much interest has been shown in the development of the ketolide class. Interestingly, this new group of erythromycin A derivatives seems to share with classical macrolides the use of an active transport system responsible for their accumulation, at least in phagocytic cells (2, 30, 31). The model of human neutrophils has widely been used to study the cellular uptake of macrolides and ketolides. We also recently developed a model of a phagocytic cell line, PLB-985, a human myeloid leukemia cell line which can undergo granulocytic differentiation under specific conditions (3). We have been able to compare all available ketolides by use of these two models (2, 3, 29–31). To extend our data on structure-activity relationships, we have investigated in the present study the accumulation of a new ketolide, ABT-773, for which no data have yet been published.

The results presented here confirm those of previous studies (29–31), suggesting that macrolide accumulation results from two mechanisms, one of which depends on the physicochemical properties of the molecules (ionization, lipid solubility, steric hindrance) which could regulate the binding affinity (and, to various degrees, passive transmembrane passage) to a membrane carrier; this carrier, which possibly exists in activated and deactivated forms, would be responsible for the main, active transport mechanism.

ABT-773 accumulated in PMNs in a time-dependent manner from 0.5 to 30 min, and then the accumulation reached a plateau. C/Es varied from about 35 (0.5 min) to 317 (30 min), with mean values of about 321, 341, and 285 at 60, 120, and 180 min, respectively. The capacity to accumulate ketolides (C/E) was significantly greater in PMNs than in ND- and D-PLB (Fig. 1B). However, the overall levels of accumulation (Fig. 1A) were similar in PMNs and D-PLB and were significantly superior to the level observed in ND-PLB, although a significant decrease was obtained at 180 min to the level at 30 min (C/Es from 26 [0.5 min] to 201 [30 min] and from 118 [180 min] for D-PLB and C/Es from 21 [0.5 min] to 129 [30 min] and from 60 [180 min] for ND-PLB); this suggests that an efflux mechanism which could take place parallel to cellular uptake becomes prominent in PLB-985 cells after extensive incubation times. HMR 3004 displayed a similar uptake kinetics profile. Whatever cells were used, ABT-773 always accumulated better than HMR 3004.

In support of our hypothesis of the presence of an efflux mechanism parallel to uptake, we observed that ABT-773 was rapidly released from loaded cells (about 9%/min in the first 5 min), and then there was a plateau owing to the equilibrium between entry and efflux. When the cells were placed in fresh drug-free medium, there was a new efflux, with less than 10% remaining associated within 30 min of incubation. The release of ABT-773 from PMNs was significantly greater than that of HMR 3004, but the efflux from PLB-985 cells was similar for the two ketolides. This difference in the efflux of the two ketolides from PMNs (and the differences in efflux between PMNs and PLB-985 cells for HMR 3004) can be explained by their different cellular locations. ABT-773 was located mainly (about 75 to 78%) in the cytosols of PMNs, D-PLB, and ND-PLB. HMR 3004 was better accumulated in the granular compartment of PMNs (65%); the percentage associated with the granules in D- and ND-PLB was less than that in PMNs (about 30%) and was similar to that obtained with ABT-773. As has already been observed with other macrolides and ketolides, verapamil strongly increased the amount of ABT-773 released from loaded cells (PMNs and PLB-985 cells). The PKA inhibitor H89 also increased the efflux of ABT-773 to a lesser extent than that of verapamil.

There is now strong evidence that an active membrane transport system plays a major role in the intracellular accumulation

of macrolides. In agreement with this hypothesis, we noted that the level of ketolide uptake by formaldehyde-killed cells was low (passive association, about 20 to 30%), and the level of accumulation was also reduced at temperatures below 37°C, without a significant increase over 180 min. The uptake was more rapid at 40°C than at 37°C. ΔG was low (about 50 kJ/mol), which agrees with the mainly cytosolic location of ABT-7733. The level of ABT-773 accumulation was significantly decreased in acidic medium; by contrast, a significant increase occurred at basic pH. This explains the enhancing and decreasing effects of certain inhibitors due to the pH of their solutions (KCN, alkaline pH; levofloxacin, acidic pH). In general, it is difficult to modify macrolide and ketolide uptake with metabolic poisons (23, 30, 31), likely because the concentrations used (1 mM), which are chosen to preserve cell viability, are insufficient to completely block cell metabolism.

Ketolide accumulation displayed a concentration-dependent profile compatible with a saturable transport mechanism. For ABT-773, the kinetics constant were similar whatever the cell type, with a V_{max} of about 10,000 ng/2.5 × 10⁶ cells and a K_m of about 60 mg/liter. In agreement with previous data, we obtained a lower K_m (about 10 to 20 mg/liter) with HMR 3004, suggesting that it has a greater affinity for the carrier, which was similar for whatever cell type was used, but also a lower V_{max}, which is compatible with the higher level of accumulation of ABT-773 in the first 5 min. The carrier system seems to be present in nondifferentiated phagocytic cell lines, but its functionality depends on the level of cell maturation, as discussed below. Roxithromycin, HMR 3004, and ABT-773 pretreatment inhibited ABT-773 uptake in a noncompetitive manner (decreased V_{max}), suggesting that the fixation of any of the drugs modified the conformation of the carrier and blocked the subsequent binding of ABT-773. By contrast, azithromycin behaved as a classical competitive inhibitor. Another explanation could be the modification (deactivation) of the activating pathway by the first three derivatives, but this hypothesis was not assessed here. We verified that cell viability was not modified by incubation in the presence of these high concentrations of macrolides and ketolides.

The effects of various PMN activators or inhibitors on the uptake of these new ketolides was also compatible with an active transport system. As previously observed with other macrolides and ketolides (29–31), ABT-773 uptake by PMNs was strongly inhibited by the PKC activator PMA, verapamil, and nickel. The effect of PMA was also demonstrated for ABT-773 uptake by D-PLB, but not by ND-PLB. Verapamil and nickel interfered with drug uptake, whatever the cell type. These data suggest that PKC-dependent phosphorylation mechanisms are involved in the optimal activity or activation of the macrolide carrier and that this pathway is not mature in nondifferentiated phagocytes. By contrast, verapamil-modified transduction systems and the Na⁺-Ca²⁺ exchanger (the target of Ni²⁺) are operating in PMNs and nondifferentiated phagocytes.

As has been observed with all erythromycin A derivatives (1, 3, 29, 31), ABT-773 displayed a concentration- and time-dependent inhibitory effect on PMN oxidative burst (Fig. 6 and Table 3). The IC₅₀s were in the same range as those obtained with HMR 3004 (29). We have previously reported that the inhibitory effects of classical macrolides are linked to the pres-
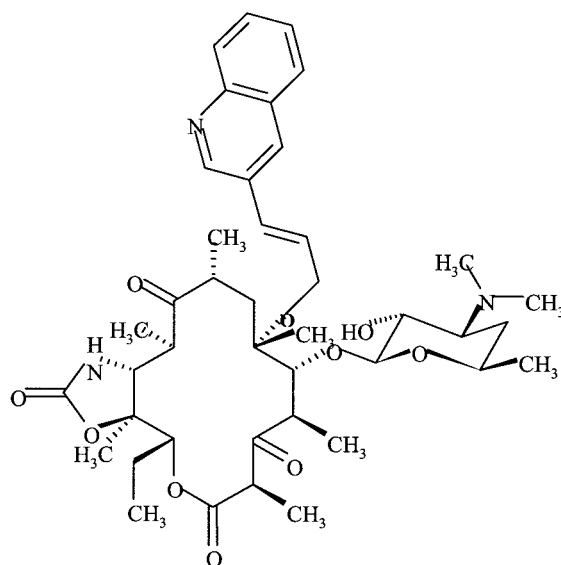

FIG. 7. Chemical structure of ABT-773.

ence of the L-cladinose at position 3 of the lactone ring (1). However, two ketolides (HMR 3004 and HMR 3647 [telithromycin]) are also endowed with inhibitory properties. For HMR 3004, the chemical structure responsible for this effect seems to be the quinoline nucleus attached to the lactone ring. ABT-773 also possesses the quinoline substituent (Fig. 7), and this likely represents the cause of the inhibitory effect. The inhibitory concentrations are larger than those reported in human plasma after a single dose (26). However, it is likely that the concentrations in tissues, where phagocytes function, and the concentrations achieved after multiple doses are higher and are compatible with those obtained here.

Even though it does seem that the development of ABT-773 has been arrested, it is important to know the antimicrobial activity, pharmacokinetics, and toxicity of this ketolide. The study reported here presents a model that can be used to better understand structure-activity relationships in the ketolide-macrolide family and to help with the selection of new molecules in this class. The cellular accumulation of macrolides and ketolides supports their intracellular bioactivities, although other criteria, such as the respective locations of the drug and the pathogen, the cellular binding and inactivation of the drug, the possible synergy between drug activity and cell defense factors, and pathogen status, must be taken into consideration for determination of their overall activities against intracellular pathogens (13, 14). Whether the intracellular (granular) location of ABT-773 impairs its intracellular bioactivity was not investigated here. Some intracellular pathogens (for instance, *Listeria*) reside in the cytoplasm, but in general, they do not belong to the macrolide spectrum. The bacterial targets of macrolides are generally located in a phagolysosome or a phagosome but are sometimes located in a phagosome with a special structure (e.g., in *Legionella*). However, it must be pointed out that ABT-773 is active against various intracellular pathogens, including *Legionella* (11), and that clarithromycin and roxithromycin, about 40 to 50% of which are located in the cytoplasm, have also been demonstrated to have excellent ac-

tivities in vitro and in vivo against a wide range of intracellular pathogens. In addition, the cellular location of ABT-773 was investigated with resting cells only and may vary when cells are stimulated, for instance, after phagocytosis.

The macrolide carrier has not yet been identified. This is a major goal for macrolide development and research to improve the intracellular activities of forthcoming macrolides, to minimize some unwanted effects (if the carrier is present on other host cells), and to develop nonantibiotic properties (for instance, anti-inflammatory or anticancer activity).

ACKNOWLEDGMENT

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