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Biochemical Pharmacology 62 (2001) 765–772

Biochemical Pharmacology

Effects of miltefosine on various biochemical parameters in a panel of tumor cell lines with different sensitivities

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Received 17 January 2001; accepted 18 May 2001

Abstract

We investigated endocytosis activity, uptake of miltefosine (hexadecylphosphocholine), phospholipid and cholesterol content, the cell cycle, and apoptosis in 13 tumor cell lines (MCF7, MCF7/ADR, KB-3-1, KB-8-5, KB-C1, HeLa, HeLa-MDR1-G185, HeLa-MDR1-V185, CCRF/CEM, CCRF/VCR1000, CCRF/ADR5000, HL-60, HL-60/AR) with different sensitivities to treatment with the antitumor phospholipid analogues miltefosine and D-21266 (octadecyl-(*N*,*N*-dimethyl-piperidino-4-yl)-phosphate). In this panel of cell lines, *MDR1* (multidrug resistance gene 1)- and MRP1 (multidrug resistance-associated protein)-expressing cells were found to be slightly more resistant to both compounds than sensitive parental cells. No correlation was found between resistance to miltefosine and endocytosis, intracellular concentration of miltefosine, the phospholipid and cholesterol content, induction of apoptosis, or cell cycle alterations in all the cell lines tested. Wild-type p53 containing WMN Burkitt's lymphoma cells and wild type p53-deficient CA46 exhibited similar sensitivities to miltefosine. The low percentage of apoptosis induced in MCF7 cells lacking caspase 3 indicated that caspase 3 seems to play an essential role in miltefosine-induced apoptosis.

Keywords: Miltefosine; Hexadecylphosphocholine; D-21266; Sensitivity; Apoptosis

1. Introduction

Phospholipid analogues are a new class of drugs that exhibit broad antineoplastic activity [1,2]. Miltefosine represents the first of these compounds used in clinical application [1,2]. In several countries, it has been approved as topical treatment of skin metastases resulting from breast cancers [3]. Its development as an oral drug was hampered by its gastrointestinal toxicity. For this reason, hexadecylphosphocholine derivatives with a better therapeutic index were sought. D-21266 was identified as a suitable candidate [4] and is currently used in clinical trials. The exact mechanism responsible for the antitumor activity of miltefosine and D-21266 is not yet known [1,2]. However, it would be important to understand why some tumors respond to treatment with these compounds and others do not.

We investigated the reasons for different susceptibilities of various human tumor cells to treatment with miltefosine or D-21266. More than one mechanism might be responsible for the different sensitivities to these compounds. Uptake of miltefosine by the cells is accomplished by endocytosis via the plasma membrane [5]. Distinct sensitivities to miltefosine treatment may be due to different membrane properties such as membrane fluidity or the phospholipid/cholesterol ratio. Alternatively, different sensitivities might be associated with increased efflux of drugs as by MDR1,

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Abbreviations: D-21266, octadecyl-(*N*,*N*-dimethyl-piperidino-4-yl)-phosphate; HeLa-WT, HeLa wild-type cells, HeLa-MDR1-G185, HeLa-WT cells transfected with a wild-type *MDR1* gene containing glycine in position 185; HeLa-MDR1-V185, HeLa wild-type cells transfected with a mutant *MDR1* gene containing valine in position 185; HL-60, human promyelocytic leukemia cells; *MDR1*, multidrug resistance gene 1; MRP1, multidrug resistance-associated protein; miltefosine, hexadecylphosphocholine; PGP, P-170-glycoprotein; and TMA–DPH, trimethylamino–diphenylhexatriene.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00715-8

MDR2, MRP, or LRP (lung resistance protein) transport proteins [6–9]. Furthermore, since it has been shown that phospholipid analogues induce apoptosis [10–12], p53 function and response to induction of apoptosis may also be responsible for differences in sensitivity. There are reports indicating that overexpression of MDR1, in addition to drug efflux, delays the apoptotic cascade in CHO (Chinese hamster ovary) cells [13], alters the expression of genes regulating apoptosis [14], and protects human CCRF/CEM and K562 tumor cells from caspase-dependent apoptosis [15]. Differences in these features between a sensitive and a resistant cell line–sometimes from different tissues–were reported previously. Here, we compared endocytosis, accumulation of miltefosine, the content of phospholipids and cholesterol, apoptosis sensitivity, and the influence of miltefosine on the cell cycle in 5 sensitive and 8 resistant cell lines derived from the sensitive lines.

2. Materials and methods

2.1. Drugs

Miltefosine and D-21266 were from ASTA Medica. A 10-mM stock solution in 20 mM Tris–HCl (pH 7.4) was used. TMA–DPH was purchased from Molecular Probes and dissolved in dimethylformamide (Sigma Chemicals). Vinblastine was from Sigma. The MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide) assay kit was obtained from Boehringer Mannheim.

2.2. Tissue culture

MCF7 (human breast adenocarcinoma) cells, the multidrug-resistant subline MCF7/ADR, CCRF-CEM (human lymphoblastoid cells), the multidrug-resistant sublines CCRF/VCR1000, CCRF/ADR5000 [16], HeLa (human epitheloid cervix carcinoma), and two multidrug-resistant sublines (HeLa-MDR1-G185, HeLa-MDR1-V185), HL-60 (human promyelocytic leukemia), and the MRP1-overexpressing HL-60/AR cells [17] were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine and 0.1 μg/mL of gentamycin. KB-3-1 cells (human oral epidermoid carcinoma) and the multidrug-resistant sublines KB-8-5 and KB-C1 [18] were grown in Dulbecco's modified Eagle's medium (4.5 g glucose/liter) and supplemented as described above.

The two multidrug-resistant MDR1-overexpressing HeLa cell lines were obtained by transfection of human HeLa S3 (HeLa-WT) cervic carcinoma cells with an MDR1 wild-type gene construct (HeLa-MDR1-G185) and with a mutation in codon 185 (gly-val) [19]. Following transfection, HeLa-MDR1-G185 cells were grown in the presence of vinblastine (100 nM) and HeLa-MDR1-V185 in the presence of colchicine (240 ng/mL). MDR1-mRNA expression was controlled by reverse transcriptase–polymerase chain reaction [20]. Wild-type and mutant genes were controlled by sequencing [20].

Every other week the following drug concentrations were added to the medium of the multidrug resistant stock cultures: MCF7/ADR: 10 μg adriamycin/mL; CCRF/VCR1000: 1 μg vincristine sulfate/mL; CCRF/ADR5000: 5 μg adriamycin/mL; HeLa-MDR1-G185: 100 nM vinblastine; HeLa-MDR1-V185: 240 ng colchicine/mL; HL-60/AR: 100 nM daunomycin/mL; KB-8-5: 10 ng colchicine/mL; KB-C1: 1 μg colchicine/mL.

Dose–response curves for calculations of the IC_{50} values and resistance factors (Table 1) were obtained by seeding the cells in 96-well plates. Following an incubation period of 4 hr, the drugs were added and cells were exposed to the drugs continuously for 72 hr. Subsequently, cell proliferation was determined by the MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide) assay [21]. The IC_{50} values were calculated by the CalcuSyn software from Biosoft.

2.3. Determination of lipids and cholesterol

Logarithmically growing cells were washed with PBS at 4°, lysed with 0.2% SDS, and lipids extracted with chloroform/methanol (2:1). Non-lipids were removed by solvent partitioning [22]. The extracts were dried under nitrogen. Lipid phosphorus [23] (as measurement of phospholipids) and cholesterol [24] were determined as described.

2.4. Endocytosis

Cells (2.10^5) were seeded in 2 mL of culture medium in a 3.5-cm dish and incubated for 16 hr. Subsequently, the cells were washed with PBS, incubated in 2 mL of fresh medium containing 10% fetal bovine serum and 2 μM TMA–DPH at 37° for 30 min. To evaluate the effect of miltefosine on endocytosis (Fig. 1B), cells were treated with doses of miltefosine corresponding to the IC_{50} values at 37° for 30 min, washed with PBS, reincubated in 2 mL medium containing 10% fetal bovine serum and 2 μM TMA–DPH at 37° for 30 min. The miltefosine-treated and untreated cells were washed three times with ice-cold PBS containing 3% fatty-acid-free BSA (Sigma Chemicals) to remove TMA–DPH from the plasma membrane, and scraped off the dish with a rubber policeman. Two hundred and fifty microliters of the cell suspension in PBS was transferred into a 96-well plate and the fluorescence intensity was quantitated with a Fluoroscan II, Version 4.0 (Bio-Rad; excitation 355 nm, emission 460 nm) at room temperature. The results (Fig. 1) are expressed as percentage of internalized TMA–DPH fluorescence intensity compared to that of the peripheral membrane measured in cells without the washing procedure with fatty-acid-free BSA immediately after addition of TMA–DPH [25].

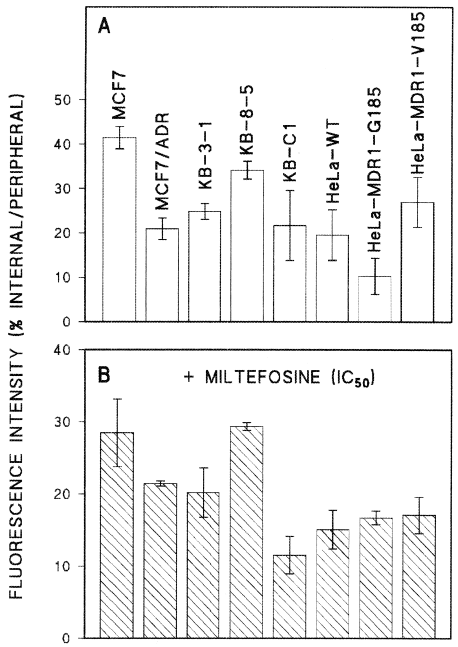

Fig. 1. Endocytosis of TMA–DPH in the absence and presence of miltefosine. Endocytosis of TMA–DPH was determined as described in Materials and methods. The cells were incubated with 20 μM TMA–DPH for 30 min. The results are expressed as percentage of internalized TMA–DPH fluorescence intensity compared to that of the peripheral membrane measured in unwashed cells immediately after addition of TMA–DPH. The means ($\pm$SD) of at least three independent experiments in which duplicate determinations were taken within each experiment are indicated. Panel A: cells treated with TMA–DPH only. Panel B: cells treated concomitantly with TMA–DPH and miltefosine concentrations of the IC_{50} values.

2.5. Uptake of miltefosine

Logarithmically growing cells (3–6 $\times$ 10^5/mL) were seeded in 6-well plates and grown for 24 hr. The culture medium was removed and the cells were treated with miltefosine (final concentration 20 μM) in fresh medium at 37° for the times indicated (Fig. 3). Following incubation, the cells were washed with 3% fatty acid-free BSA (dissolved in PBS) and 3 times with PBS at 4°, and lysed with 0.2% SDS. For determination of intracellular miltefosine, lipids were extracted as described above (Section 2.3).

Concentrations of miltefosine from cellular lipid extracts were measured using a specific HPLC–MS/MS method [26]. After drying under nitrogen (as described above in Section 2.31) the lipid extracts were reconstituted in 750 μL of a 50% aqueous solution of methanol (v/v) (Merck). To each vial, 25 μL of an 8 μg/mL solution of the structural miltefosine analogue D-21266 dissolved in 50% aqueous methanol was added as an external standard. Each vial was vortexed for approximately 10 sec. The calibration curve was prepared by adding standard solutions of miltefosine to the cells, giving a linear range from 2.10^{-8} mol/L to 2.10^{-2} mol/L.

HPLC was carried out on a ChromSpher (Chrompak®) 5 SI, 100 mm $\times$ 3 mm glass column at a flow rate of 0.5 mL/min with 40% CH_3CN/30% methanol/30% 8 mM ammonium acetate (v/v/v). The results were analysed by TurboIon-Spray™–HPLC/mass spectrometry (TISP-HPLC/MS/MS), with a turbo heater on an API 300 triple quadruple mass spectrometer (Perkin Elmer Sciex). Mass spectrometric analysis was performed in the positive ion SRM mode (selected reaction monitoring) using nitrogen (5.0 grade) as collision gas. The precursor-to-product ion transitions for miltefosine were selected from m/z 408.2 to 124.8. The precursor and product ions for the internal standard (D-21266) were selected at m/z 462.4 and 112.0. Each ion was monitored with a dwell time of 200 msec., with a pause time of 2 msec. Data were acquired by the PE-Sciex software (system version 1.2 and 'Sample-Control' version 1.2) and peak areas measured using PE-Sciex quantification software (MacQuan, version 1.4).

2.6. Cell cycle and apoptosis

Cell cycle analysis was performed as described previously [27]. Twenty-five thousand HeLa or MCF7 cells, 100,000 CCRF or HL-60 cells were seeded per well (2 mL) in 24-well plates. The cells were treated with the indicated concentrations of miltefosine and incubated for 24, 48, or 72 hr. In the case of HeLa and MCF7 cells, first the medium and 1 mL PBS from washing the cells were collected. Then, the cells were incubated with 300 μL of 0.5 g/L of trypsin and 0.2 g/L of EDTA in PBS for approximately 10 min. Subsequently, the cells in trypsin/EDTA were united with the previously collected medium and wash. The cells were centrifuged (150 *g*) for 15 min. CCRF and HL-60 cells were centrifuged and washed with PBS. The pelleted cells were resuspended in 200 μL PBS, 50 μL of 250 μg/mL of propidium iodide, 1% Triton X-100, and 10 μL of RNase A (10 mg/mL) and incubated at room temperature for 1 hr. Cell cycle analysis was performed by a FACscan (fluorescence-activated cell sorter, Becton Dickinson); at the same time, the percentage of apoptotic cells was quantitated based on the hypodiploid DNA peak as described by Nicoletti *et al*. [28].

3. Results

3.1. Cells exhibiting different sensitivities to miltefosine and D-21266

The resistance of parental and multidrug-resistant sublines to vinblastine, adriamycin, miltefosine, and D-21266 is shown in Table 1. It has been demonstrated previously that the resistant sublines shown in Table 1 overexpress MDR1 [20,29], with the exception of HL-60/AR cells which overexpress MRP1 [17]. All the resistant sublines including the MRP1-overexpressing HL-60/AR exhibit modest cross-resistance (1.3- to 9.6-fold) to miltefosine and D-21266 (1.8- to 11.2-fold). In MDR1-expressing cells the

Table 1
Resistance factors of the different cell lines used

Cell line	Vinblastine	Adriamycin	Miltefosine	D-21266
MCF7	1	1	1	1
MCF7/ADR	**21.2**	**8.7**	**2.0**	**1.8**
KB-3-1	1	1	1	n.d.
KB-8-5	**15.9**	**8.3**	**1.3**	n.d.
KB-C1	**37.3**	**55.1**	**1.8**	n.d.
HeLa-WT	1	1	1	1
HeLa-MDR1-G185	**34.3**	**111.0**	**8.3**	**10.6**
HeLa-MDR1-V185	**12.5**	**69.6**	**9.6**	**7.1**
CCRF/CEM	1	1	1	1
CCRF/VCR1000	**246.0**	**67.5**	**5.9**	**4.0**
CCRF/ADR5000	**1210.0**	**39.1**	**7.4**	**11.2**
HL-60	1	1	1	1
HL-60/AR	**2.6**	n.d.	**2.7**	**3.8**

The resistance factor was obtained by IC_{50}-resistant cells/IC_{50}-sensitive cells (n.d. = not detected).

resistance to miltefosine and D-21266 was less pronounced than that to vinblastine. MRP1-overexpressing HL-60/AR cells exhibited resistance to miltefosine (2.6-fold) and D-21266 (3.8-fold) to a similar extent as to vinblastine (2.5-fold; Table 1). The resistance of the sublines to miltefosine was previously found to be correlated to the expression of MDR1, with PGP binding and transporting miltefosine [30]. Thus, the question arises as to whether the phenomenon of resistance can be linked to the actual intracellular concentrations of miltefosine.

3.2. Endocytosis and miltefosine uptake

Miltefosine exhibits high affinity for the plasma membrane and after endocytosis, it can be localized in intracellular membranes [5]. We investigated whether the differences in the sensitivity of these cell lines to miltefosine correlate with the intracellular accumulation of the compound. For measuring endocytosis in the sensitive versus the resistant cells, we used the membrane fluorescence probe TMA–DPH. This compound interacts with living cells by instantaneous partition between the external medium and the plasma membrane, where it becomes fluorescent. The corresponding fluorescence intensity is then proportional to the cell surface. Once incorporated into the plasma membrane, it follows this membrane in the consecutive intracellular traffic and monitors endocytosis [25]. Because this endocytosis assay is performed differently in adherent and suspension cells, we used only adherent cells and omitted the CCRF and HL-60 cell lines. In sensitive MCF7 cells endocytosis was higher than in the resistant MCF7/ADR cells (Fig. 1A). The sensitivities of the three KB sublines were rather similar (Table 1). Endocytosis was also similar to KB-3-1 and KB-C1 cells. Compared with these cell lines, it was higher in KB-8-5 cells. In one resistant HeLa subline (HeLa-MDR1-G185) endocytosis was lower, while in the other resistant subline (HeLa-MDR1-V185) it was higher than in the sensitive parental line (Fig. 1A). In presence of IC_{50} concentrations of miltefosine, endocytosis was also not correlated with the IC_{50} values of miltefosine (Fig. 1B). These results suggest that the level of endocytosis does not explain the differential resistance to miltefosine.

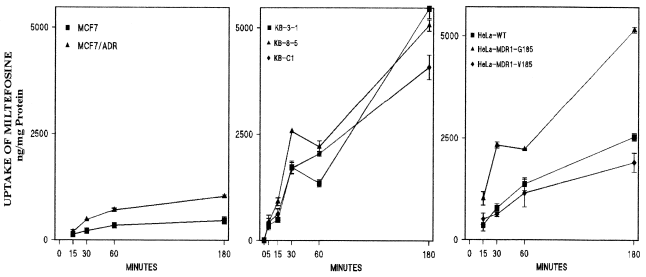

Fig. 2. Uptake of miltefosine. The cells were incubated with 20 μM of miltefosine for the indicated times. Subsequently, the lipid fraction of the cells was isolated. Miltefosine was determined by HPLC and mass spectrometric analysis. The data represent the means (± SD) of two different experiments in which duplicate determinations were taken within each experiment.

Quantification of endocytosis by TMA–DPH was performed following an incubation period of 30 min. In order to obtain information about post-endocytotic events, we measured the uptake of miltefosine by HPLC analysis in adherent cells following incubation periods up to 180 min. As shown in Fig. 2, the uptake of miltefosine was quite low in MCF7 and MCF7/ADR cells compared to KB or HeLa cells. The miltefosine uptake was slightly higher in the resistant MCF7/ADR subline compared with the sensitive line. In the KB cell lines, the IC_{50} values for miltefosine were low (KB-3-1 = 2.5 μM, KB-8-5 = 3.2 μM, KB-C1 = 4.5 μM) compared to MCF7 (MCF7 = 34.6 μM, MCF7/ADR = 69.8 μM) and HeLa cells (HeLa-WT = 6.8 μM, HeLa-MDR1-G185 = 57 μM, HeLa-MDR1-V185 = 65 μM). The uptake of miltefosine in KB cells was high (Fig. 2), indicating that in the KB cell lines the low IC_{50} value is the result of a high uptake. Despite significantly higher drug uptake in HeLa-MDR1-G185 cells compared to MCF7 and MCF7/ADR cells, the IC_{50} values of MCF7/ADR (IC_{50} = 69.8 μM) and HeLa-MDR1-G185 (IC_{50} = 57 μM) were in the same range. Sensitive HeLa wild-type and resistant HeLa-MDR1-V185 showed similar uptake, whereas the uptake of miltefosine was higher in resistant HeLa-MDR-G185 (Fig. 2). These results suggest that the intracellular concentration of miltefosine is not directly related to the sensitivity.

3.3. Phospholipid and cholesterol content

One possible explanation for the resistance of tumor cells to phospholipid analogues are PGP-mediated phospholipid

Table 2
Phospholipid and cholesterol content

Cell line	Phospholipids %	Cholesterol %
MCF7	100.0	100.0
MCF7/ADR	**69.3 ± 13.0**	**63.4 ± 6.3**
KB-3-1	100.0	100.0
KB-8-5	**97.1 ± 2.7**	**116.8 ± 10.2**
KB-C1	**110.6 ± 22**	**135.3 ± 5.1**
HeLa-WT	100.0	100.0
HeLa-MDR1-G185	**102.8 ± 30.0**	**116.8 ± 30.6**
HeLa-MDR1-V185	**97.9 ± 13.6**	**91.2 ± 12.7**
CCRF/CEM	100.0	100.0
CCRF/VCR1000	**98.9 ± 11.1**	**100.0 ± 13.8**
CCRF/ADR5000	**125.0 ± 41.0**	**193.0 ± 26.9**
HL-60	100.0	100.0
HL-60/AR	**119.5 ± 26.2**	**137.9 ± 27.0**

Phosphorus was determined in the lipid extracts as measure for the amount of phospholipids. Cholesterol and phosphorus were quantified as described in Materials and methods. The nmol of phosphorus and cholesterol per 10^6 drug-sensitive cells were taken as 100%. Relative increases or decreases in resistant cell lines (in bold) are shown.

Table 3
P/C ratio in the different cell lines

Cell line	P/C ratio
MCF7	1.83
MCF7/ADR	**2.00**
KB-3-1	2.80
KB-8-5	**2.46**
KB-C1	**2.24**
HeLa-WT	1.78
HeLa-MDR1-G185	**1.78**
HeLa-MDR1-V185	**1.52**
CCRF/CEM	1.60
CCRF/VCR1000	**1.72**
CCRF/ADR5000	**1.14**
HL-60	2.90
HL-60/AR	**2.53**

The P/C ratio was obtained by phospholipids (in nmol per 10^6 cells) divided by cholesterol (in nmol per 10^6 cells). The means of at least three independent experiments in which duplicate determinations were taken within each experiment are shown. The resistant cell lines are in bold letters.

or cholesterol alterations in the membranes [31]. It has been reported that the enrichment of HL-60 cells in cholesterol resulted in resistance to the phospholipid analogue ET-18-OCH_3 (1-*O*-octadecyl-2-*O*-*rac*-glycero-3-phosphocholine) [32]. Therefore, we quantitated the phospholipids and cholesterol in our set of cell lines. Table 2 shows that resistant KB-C1, CCRF/ADR5000, and HL-60/AR cells contained more, whereas resistant MCF7/ADR contained less phospholipids compared to their corresponding sensitive sublines. The cholesterol content in resistant MCF7/ADR and HeLa-MDR1-V185 was lower, while in resistant KB-8-5, KB-C1, HeLa-MDR1-G185, CCRF/ADR5000, and HL-60/AR it was higher than that in the sensitive controls (Table 2). Furthermore, the phospholipid/cholesterol ratio did not correspond to the resistance to vinblastine, miltefosine, or D-21266. The ratio was enhanced in resistant MCF7/ADR and CCRF/VCR1000, and reduced in KB-8-5, KB-C1, CCRF/ADR5000 and HL-60/AR compared to the parental sensitive cell lines (Table 3). Membrane fluidity quantitated by TMA–DPH anisotropy also did not correlate with the resistance to vinblastine, miltefosine, or D-21266 (data not shown). Thus, the results suggest that the phospholipid and cholesterol content of the cells or the phospholipid/cholesterol ratio are not the determining factors for resistance to miltefosine or D-21266.

3.4. Cell cycle analysis

It has been shown previously that the phospholipid analogue ilmofosine arrests Burkitt's lymphoma cells in the G_2 phase of the cell cycle by suppression of cdc2 kinase activation [33]. Analysis of the cell cycle after treatment with 10 μM miltefosine showed no effects that could be correlated to the resistance of the MCF7, KB, and HL-60 cell lines (data not shown). An increase in the G_2 populations in the sensitive CCRF and HeLa compared to the resistant sublines was observed (Fig. 3). Thus, sensitivity to miltefosine cannot be related to changes in cell cycle regulation. The previously reported arrest of cells in the G_2 phase of the cell cycle [33] cannot be regarded as a general phenomenon induced by phospholipid analogues.

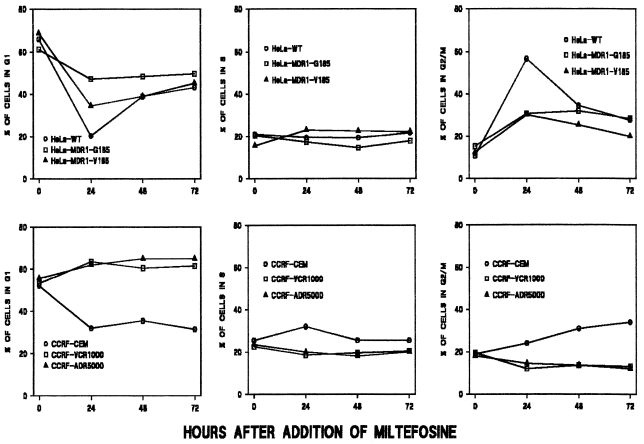

Fig. 3. Effect of miltefosine on cell cycle progression. Cell cycle analysis was performed as described in Materials and methods following treatment with 10 μM miltefosine for the indicated times. The means of two different experiments in which duplicate determinations were taken within each experiment are shown.

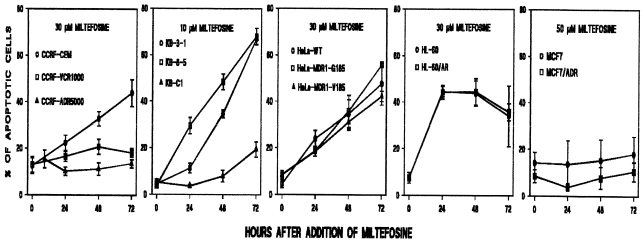

Fig. 4. Quantification of apoptosis following miltefosine treatment. The cells were treated with miltefosine for the times indicated. Apoptosis was determined as described in Materials and methods. The means of at least three independent experiments (±SEM) in which duplicate determinations were taken are shown.

3.5. Apoptosis

Phospholipid analogues have the ability to induce apoptosis [10–12]. Differential induction of apoptosis might possibly explain the phenomenon of drug resistance in the cell lines investigated. p53 is one major factor influencing apoptosis by antitumor treatment. For this reason, we compared the effects of miltefosine in p53 wild-type WMN Burkitt's lymphoma cells with CA46 Burkitt's lymphoma cells in which one allele of p53 is deleted and the second one is mutated. The IC_{50} values of p53 wild-type WMN cells and CA46 p53 mutants for miltefosine were found to be 16.11 μM (±4.1 SD, N = 7) and 14.33 μM (±0.6, N = 6), respectively. Thus, deletion or mutation of p53 was not the cause of resistance to miltefosine, because p53 wild-type WMN cells are equally sensitive as p53 mutant CA46 cells.

Differential susceptibility to apoptosis could explain the drug uptake-independent sensitivity to miltefosine. Treatment with miltefosine was associated with a decreased apoptosis induction in the multidrug-resistant CCRF and KB sublines compared to their matched sensitive cell lines (Fig. 4). No significant differences could be observed, however, when comparing the sensitive and resistant HeLa and HL-60 cell lines. Both, the sensitive and the multidrug-resistant MCF7 cell lines were equally refractory to apoptosis induction by 50 μM of miltefosine (Fig. 4). Even increasing the miltefosine concentration to 100 μM miltefosine did not induce apoptosis in MCF7 cells (data not shown). The low levels of apoptotic cells in miltefosine-treated MCF7 cell lines seems to be due to the lack of caspase 3 in these cells [34], indicating that apoptosis induced by miltefosine is dependent on caspase 3 and that expression of MDR1 or MRP1 does not lead to a reduction in apoptosis. Also transfection of the *MDR1* gene into HeLa cells did not lead to altered apoptosis sensitivity. The differences in apoptosis sensitivity of resistant versus sensitive CCRF and KB cells are probably not caused by MDR1 expression, but are rather the result of co-selection of cells for apoptosis resistance during the selection procedure for drug resistance.

4. Discussion

The underlying mechanism of the antiproliferative activity of phospholipid analogues in tumor cells is not known at present. We tested several biochemical parameters for their possible correlation with resistance to miltefosine, namely the intracellular drug concentrations, alterations in lipid composition, apoptosis sensitivity, or the regulation of the cell cycle. Comparisons of cells with different sensitivities derived from different tissues have the disadvantage that the sensitivities may be influenced by cell type-specific factors. In comparisons of parental cells with resistant sublines these cell type differences are avoided. We employed five different parental human tumor cell lines and the corresponding resistant sublines comprised in all 13 cell lines. MDR1- or MRP1-expressing cell lines are less sensitive to miltefosine and D-21266, as miltefosine binds to and is transported by PGP [30]. Thus, the intracellular concentration of miltefosine should be responsible for the sensitivity to the compounds.

However, the intracellular concentration of miltefosine in both MCF7 cell lines was lower than in KB cells (Fig. 2), corresponding to higher IC_{50} of MCF7 and the low IC_{50} of KB cells. Previously Fleer *et al.* [35] reported an unusual sensitivity of KB cells to miltefosine. We observed, however, a higher endocytosis activity in resistant KB-8-5 and HeLa-MDR1-G185 cells than in the sensitive parental lines (Fig. 1). The uptake experiments also show higher concentrations of miltefosine in resistant MCF7/ADR and HeLa-MDR1-G185 compared to the sensitive parental cells (Fig. 2). Thus, the accumulation of miltefosine cannot be the only determining factor for sensitivity. This is in agreement with a previous report showing that uptake and intracellular accumulation of miltefosine are not the determinants of resistance to this compound [36]. Therefore, we investigated additional parameters that could be responsible for the resistance differences.

It has been shown previously that phospholipid analogues induce apoptosis [10–12]. Differences in the status of p53 also do not seem to be responsible for an altered sensitivity to miltefosine because p53 wild-type Burkitt's lymphoma cells were similar sensitive as p53 mutant cells. It has also been reported that overexpression of PGP delays the apoptotic cascade in CHO (Chinese hamster ovary) fibroblasts [13], leads to altered expression of genes regulating apoptosis in human myeloid leukemia cells [14], and protects CCRF/CEM and K562 cells from caspase-dependent apoptosis [15]. In the latter report, the question was raised whether this apoptosis resistance is to be found only in cells in which the resistance was induced with antitumor drugs, or also in cells transfected with MDR1. Our results show that in HeLa cells transfected with MDR1, the percentage of apoptotic cells following treatment with miltefosine is about the same as in non-transfected HeLa cells (Fig. 4). If indeed MDR1 expression were responsible for reduced apoptosis sensitivity, MDR1-expressing cells

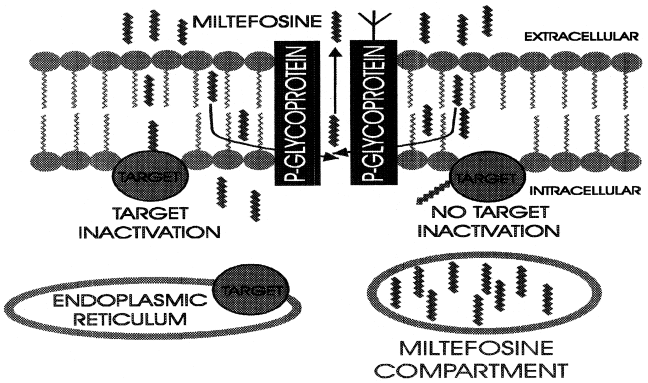

Fig. 5. Speculative representation of the possible action of miltefosine. PGP may remove miltefosine from the plasma membrane like a vacuum cleaner. Thus, miltefosine might not be able to affect a target localized in the plasma membrane. If the target–drug interaction site is in the membrane and not in the cytosol, neither would the intracellular miltefosine be able to interact with the active site of the target. Alternatively, intracellular miltefosine could be kept in a certain compartment and, therefore, be unable to reach a target such as cytidylyl transferase, which is located in the endoplasmic reticulum.

would also, at least in part, exhibit resistance to drugs not transported by PGP, as for example cisplatin. However, the HeLa sublines transfected with MDR1 constructs did not exhibit different sensitivities to cisplatin (data not shown).

Here, we show that miltefosine induces apoptosis in CCRF, KB, HeLa, and HL-60 cells (Fig. 4). Due to the lack of caspase 3 in MCF7 breast carcinoma cells [33], the level of apoptosis induced by miltefosine is rather low (Fig. 4). This suggests that miltefosine-induced apoptosis is dependent on caspase 3. Beside reduced uptake, resistance to apoptosis may be a reason for the high IC_{50} in MCF7 cells. A comparison between the sensitive and resistant sublines shows a difference in the percentage of apoptotic cells in KB and CCRF cells (Fig. 4). It is very likely that these differences originate from the selection by resistance-inducing drugs. The similar apoptosis in sensitive and resistant HeLa and HL-60 cells shows that at least in these cells different apoptosis sensitivity is not the cause of differences in drug sensitivity.

Analysis of the phospholipid and cholesterol content and of the cell cycle did not reveal any differences that could be correlated with drug resistance. If the induction of apoptosis and the amount of miltefosine in the cell are not major determinants for differences in drug sensitivity, how can drug efflux by PGP lead to an increase in the resistance? Fig. 5 shows a schematic representation of a possible mechanism. If PGP functions as a flippase [6,37,38], it may remove miltefosine from the membrane like a vacuum cleaner. Therefore, the compound may not reach the target even if it is located in the membrane. If the target is situated in the membrane in a manner that the site for interaction with miltefosine cannot be approached by miltefosine from the cytosol, a high concentration of the drug in the cytosol may not be able to influence the sensitivity. It has been reported previously that phospholipid analogues inhibit protein kinase C [39], cytidylyl transferase (phosphatidylcholine biosynthesis) [40], and phospholipases [41–43]. All these proteins with the exception of cytidylyl transferase are located in the plasma membrane or are translocated to the plasma membrane at least for certain periods of time. Miltefosine may inhibit the target directly or may interfere with the translocation of the target. Compartmentalization of miltefosine could prevent the interaction with cytidylyl transferase which is located in the cytosol and in the endoplasmic reticulum. Our results show that none of the parameters analyzed, namely endocytosis, the intracellular concentration of miltefosine, the phospholipid or cholesterol content of the cells, cell cycle alterations, p53 mutation, or induction of apoptosis by miltefosine can, on their own, determine the sensitivity to miltefosine in all cell lines.

Approximately 40 to 50% of primary breast carcinomas express MDR1 [44]. Expression of MRP1 has also been reported in breast cancer [45]. The results reported here illustrate that expression of these drug transporters leads to resistance to miltefosine and D-21266. Even if the resistance to these compounds is not as pronounced as to vinblastine (Table 1), it may suffice to reduce the antitumor activity of miltefosine and D-21266.

Acknowledgment

This work was supported by Grant P10664-MED from the Austrian Science Fund.

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