Resistance to the new anti-cancer phospholipid ilmofosine (BM 41 440)

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Summary The thioether phospholipid ilmofosine (BM 41 440) is a new anti-cancer drug presently undergoing phase II clinical trials. Because resistance to anti-tumour drugs is a major problem in cancer treatment, we investigated the resistance of different cell lines to this compound. Here we report that the multidrug-resistant cell lines MCF7/ADR, CCRF/VCR1000, CCRF/ADR500, CEM/VLB, WT and HeLa cell lines transfected with a wild-type and mutated (gly/val185) multidrug resistance 1 gene (MDR1) are cross-resistant to ilmofosine compared with the sensitive parental cell lines. In CEM/VM-1 cells, in which the resistance is associated with an altered topoisomerase II gene, no cross-resistance to ilmofosine was observed. Ilmofosine is not capable of modulating multidrug resistance and neither does it reduce the labelling of the P-glycoprotein (P-gp) by azidopine nor alter ATPase activity significantly. The resistance to ilmofosine in multidrug-resistant CCRF/VCR1000 cells cannot be reversed by the potent multidrug resistance modifier dexniguldipine-HCl (B8509-035). A tenfold excess of ilmofosine does not prevent the MDR-modulating effect of dexniguldipine-HCl. Treatment of cells with ilmofosine does not alter the levels of MDR1 mRNA. Long-term treatment of an ilmofosine-resistant Meth A subline with the drug does not induce multidrug resistance, indicating that ilmofosine does not increase in the absence of P-gp. Determination of the MDR2 mRNA levels in the cells revealed that the resistance pattern to ilmofosine is not correlated with the expression of this gene. It is concluded, therefore, that multidrug-resistant cells are cross-resistant to ilmofosine and that the compound is not a substrate of Pgp. No association between the expression of the MDR2-encoded P-gp and resistance to ilmofosine was observed. It is supposed that MDR1-associated alterations in membrane lipids cause resistance to ilmofosine.

Keywords: ilmofosine; BM 41 440; multidrug resistance; MDR1; MDR2

A major problem in the successful treatment of tumours by chemotherapy is the selection of tumour cell populations with intrinsic or acquired resistance to anti-cancer drugs. To develop drugs that do not exhibit cross-resistance to that of other compounds or that reverse resistance, it is essential to understand the mechanisms responsible for antiproliferative activity and for treatment failure. Phospholipid analogues are a new class of drugs that exhibit broad antineoplastic activity (Berdel, 1991). Miltefosine (hexadecylphosphocholine) is a licensed anti-cancer drug for the topical treatment of skin metastases resulting from breast cancers and lymphomas. For ilmofosine (BM 41 440, 3-hexadecyl-mercaptop-2-methoxymethyl-propyl-1-phosphocholine), clinical phase II trials for the treatment of several tumours are currently under way (Berdel, 1991; Winkelmann et al., 1992). Although these drugs are used in the clinic for treatment of patients, the mechanism of action is not fully understood. It has been reported that phospholipid analogues interfere with normal phospholipid metabolism, inhibit the binding of the epidermal growth factor, inhibit protein kinase C and phospholipase C and suppress the activation of cdc2 (Hofmann et al., 1989; Berdel, 1991; Powis et al., 1992; Hofmann et al., 1994). When considering the clinical use of phospholipid analogues, the following questions need to be asked: (1) why are cells refractory to these compounds; (2) can cells acquire resistance; and (3) is there cross-resistance to drugs whose efficacy has been limited by the development of resistance? The purpose of this investigation was to obtain additional information about resistance to ilmofosine. In experimental systems, resistance to this new compound has been observed (Himmelmann et al., 1990; Herrmann, 1985; Petersen et al., 1992). One mechanism causing resistance to a variety of anti-tumour compounds is the so-called multidrug resistance, caused by increased drug efflux frequently associated with the expression of a 170-kDa glycoprotein (P-gp) encoded by the MDR1 gene (Gottesman and Pastan, 1993). This transporter exports preferentially lipophilic compounds (Gottesman and Pastan, 1993). Thus, P-gp-dependent multidrug resistance (MDR) could influence the sensitivity to ilmofosine. Cross-resistance of multidrug-resistant mouse P388/ADR and sarcoma S180/ADR cell lines to ilmofosine has been observed previously (Himmelmann et al., 1990).

The MDR genes are members of a small, highly conserved family that comprises two members in humans, MDR1 and MDR2 (Thorgerisson et al., 1991). Recently, it was reported that the MDR2-encoded P-glycoprotein has an essential role in the translocation of phosphatidylcholine (Smit et al., 1993; Ruettz et al., 1994). Ilmofosine is a phosphatidylcholine analogue in which the long chain alkyl group is linked by a thioether bond and the β-hydroxyl group of the glycerol has been replaced by a methoxymethyl moiety. Because ilmofosine is an analogue of phosphatidylcholine, it may also be transported by the MDR2-encoded P-glycoprotein. Thorgerisson et al. (1991) proposed that the MDR1 and MDR2 genes are co-expressed. The cross-resistance of multidrug-resistant cells to ilmofosine (Himmelmann et al., 1990) may result from the co-expression of the MDR2 gene in multidrug-resistant, MDR1-overexpressing cells.

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Comprehensive studies concerning the role of MDRI and MDR2 in the resistance to imidofosine are still lacking. In view of the possible relevance for cancer treatment, it was decided to investigate the association of the expression of the MDRI and MDR2 genes with cross-resistance to imidofosine.

**MATERIALS AND METHODS**

**Drugs**

Doxorubicin, vinblastine, vincristine and colchicine were purchased from Sigma Chemicals, Munich, Germany. Doxorubicin and colchicine were dissolved in distilled water, vinblastine and vincristine sulphate in 0.9% sodium chloride. Imidofosine was obtained from Boehringer Mannheim, Mannheim, Germany, dissolved in 20 mM Tris-HCl, pH 7.4, and stored at +4°C. Dexniguldipine-HCl (B859-35; Hofmann et al, 1992) was obtained from Byk-Gulden, Konstanz, Germany, and dissolved in dimethylsulphoxide in glassware. The final concentration of the solvent in treated cultures and controls did not exceed 0.1%, and this was non-toxic.

**Cell lines**

The cell lines used in the experiments were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin in 5% carbon dioxide. To the medium of the multidrug-resistant cell lines CCRF/VCRI000 (Kimmig et al, 1990), 1 µg of vincristine sulphate ml⁻¹, to CCRF/ADR5000 (Kimmig et al, 1990) 5 µg ml⁻¹ doxorubicin, to CEM/VLB₉₀ (Beck et al, 1979) 100 ng ml⁻¹ vinblastine, to HeLa-MDR1-G185 100 ng vinblastine and to HeLa-MDR1-V185 cells 240 ng ml⁻¹ colchicine were added to stock cultures every second week. The multidrug-resistant MDRI-overexpressing cell lines were obtained by transfection of human HeLa S3 (HeLa-WT) cervix carcinoma cells with a MDRI wild-type gene construct (HeLa-MDR1-G185) or with a mutation in codon 185 (Gly–Val, kindly provided by Dr M M Gottesman, HeLa-MDR1-V185) (Kane et al, 1989). Following transfection, HeLa-MDR1-G185 were grown in the presence of vinblastine (100 nM) and HeLa-MDR1-V185 in the presence of colchicine (240 ng ml⁻¹). One clone of each cell line was taken for further cultivation. The wild-type and mutant genes were controlled by sequencing. The resistance pattern to vinblastine and colchicine also reflect the expression of the wild-type and mutant MDRI gene. In atypical multidrug-resistant CEM/VL1-V1 cells, the resistance is associated with an altered topoisomerase II gene (Danks et al, 1988). A Meth A subline resistant to imidofosine (MR) was obtained from the parental sensitive cells (MS) as described (Herrmann, 1985). The resistant subline was grown in the presence of 6 µg ml⁻¹ imidofosine, except at the time of the experiments.

**Inhibition of cell proliferation**

Dose–response curves for CCRF/CEM, CEM/VL1, CEM/VLB₉₀, CCRF/VCRI000, CCRF/5000, MR and MS cells were established by the addition of drugs at concentrations indicated in the figures. Following incubation in the presence of the drugs for 72 h, the cells were counted with an electronic counter (Coulter-Electronics, Luton, UK). Cellular multiplication (M) was calculated by \( M = \frac{(T-T_0)(C_t-C_0)}{100} \times 100 \), where \( C \) are untreated controls and \( T \) are drug-treated cells; 0 and \( t \) equal the number of cells at time 0 and \( t \) (72 h) respectively.

**MCFC, MCF/ADR, HeLa-WT, HeLa-MDR1-G185 and HeLa-MDR1-V185 cells were plated in 96-well plates. Two hours after plating of the cells, drugs were added to the cells as indicated in the figures and exposed continuously for 72 h. Subsequently, cell proliferation was detected using the sulforhodamine B assay (Skehan et al, 1990).**

**Accumulation of rhodamine 123**

Logarithmically growing CCRF-ADR5000 cells were washed with phosphate-buffered saline and resuspended in 1 ml (5 × 10⁶ cells ml⁻¹) of Dulbecco’s modified Eagle medium without serum, supplemented with 20 mM 3-N-morpholinopropanesulphonic acid. The cells were incubated with dextraniguldipine-HCl, imidofosine or a combination of both drugs at 37°C for 30 min. After addition of 60 µl (5 µg ml⁻¹) of rhodamine 123 per ml of cell suspension, fluorescence (excitation at 488 nm) was detected by a flow cytometer

| Cell line | IC₅₀ values (resistance factor) |
|-----------|--------------------------------|
|           | Vinblastine (µM) | Doxorubicin (µM) | Imidofosine (µM) |
| MCF-7     | 5.5              | 155.4            | 7.2             |
| MCF-7/ADR | 116.9 (21.25)    | 1352.8 (8.70)    | 51.4 (7.13)     |
| CCRF/CEM  | 2.0              | 21.6             | 2.8             |
| CEM/VL1   | 169.7 (84.85)    | 560.0 (25.90)    | 8.3 (2.96)      |
| CCRF/VCRI000 | 492.0 (246.00) | 1459.0 (67.54)  | 19.9 (7.10)     |
| CCRF/ADR5000 | 2420 (1210.00) | 846.0 (39.16)   | 14.3 (5.10)     |
| HeLa-WT   | 7.3              | 231.0            | 9.9             |
| HeLa-MDR1-G185 | 251.0 (34.38) | 25650.0 (111.03) | 45.5 (4.59)     |
| HeLa-MDR1-V185 | 91.9 (12.58) | 16080.0 (69.61) | 34.1 (3.44)     |

IC₅₀ was obtained from dose–response curves to the drugs as described in Materials and methods. The mean of three independent experiments, in which duplicate samples were taken within each experiment, is indicated. The resistance factor (in brackets), indicating the resistance compared with the parental cell line, is calculated by IC₅₀-resistant/IC₅₀-sensitive.

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(FACStar, Becton Dickinson, Mountain View, CA, USA) at the times indicated in Figure 3. Emission was observed with a 530/30-nm filter and fluorescence intensity was expressed as the mean of 5000 cells gated by forward- and side-scattered light to measure only viable and single cells (Hofmann et al, 1992).

Photoaffinity labelling with [3H]azidopine
Membranes from CCRF/CEM and CCRF/ADR5000 cells (Kimmig et al, 1990) were prepared according to Hamada and Tsuruo (1988). The protein concentration was determined using an assay kit from Pierce (Rockford, IL, USA). The experiments were performed under dim light. Thirty micrograms of protein was incubated with 0.7 µCi [3H]azidopine (49 Ci mmol⁻¹, Amersham, Little Chalfont, UK) in the presence or absence of ilmofosine in a final volume of 35 µl of phosphate buffer (40 mm, pH 7.4) at room temperature for 60 min. Subsequently, the reaction mixture was placed on ice and irradiated with a UV lamp (CAMAG, Merck, Darmstadt, Germany) at a distance of 8 cm for 20 min. Samples were separated on a 4-15% polyacrylamide gel containing sodium dodecyl sulphate (SDS) and exposed to a radiographic film.

Measurement of ATPase activity
ATPase activity of P-glycoprotein partly purified from CCRF-ADR5000 cells was quantified as described (Doige et al, 1992). Ouabain (2 mm) and EGTA (1 mm) was included in the assay buffer to inhibit Na⁺/K⁺- and Ca⁺⁺-ATPases respectively. Verapamil was used as activating control and vanadate as inhibiting control.

Detection of MDR1 and MDR2 mRNA levels
For detection of the mRNA levels, total RNA was isolated using RNAzol (Biotexcs Laboratories, Houston, TX, USA). Synthesis of cDNA and amplification of the MDR1 mRNA by polymerase chain reaction was performed as described (Noonan et al, 1990). Primers for the amplification of the MDR2 mRNA were: 2061-2083 (5'-TGT CAG AAG AGC CTT GAT GTG G-3') and 2193-2215 (5'-TGG CAA TGG CAC ATA CTG TTC C-3'). Thirty cycles were performed with a denaturation temperature of 94°C (35 s), an annealing temperature of 57°C (30 s) and an extension temperature of 73°C (1 min). Starting with cycle 16, the time for synthesis was extended (5 s per cycle). β-Microglobulin was used to control the correct amount of RNA in the experiments (Noonan et al, 1990). The reaction products were separated on a 10% polyacrylamide gel and stained with ethidium bromide.

RESULTS
Effects of ilmofosine on multidrug-resistant cells
As shown in Table 1, MDR1-overexpressing MCF-7/ADR cells are cross-resistant to ilmofosine to a similar extent as to doxorubicin but less cross-resistant to vinblastine, compared with the parental

Figure 1 Concomitant treatment of CCRF/CEM and CCRF/VC1000 (A) and HeLa-WT and HeLa-MDR1-V185 cells (B) with doxorubicin and ilmofosine. The cells were exposed to the indicated concentrations of drug or drug combinations for 72 h. Experiments were performed as described in Materials and methods. Inhibition of cell proliferation is indicated as per cent of untreated controls (= 100%). Data represent the mean (± s.e.m.) of three independent experiments, in which duplicate samples were taken within each experiment.
cell line. CEM/VLB0, CCRF/VCR1000 and CCRF/ADR5000 cells exhibit modest cross-resistance to ilmofosine (Table 1). In CEM/VM-1 cells, multidrug resistance is not due to expression of MDR1 but is associated with an alteration in the topoisomerase II gene (Danks et al, 1988). These cells are resistant to the topoisomerase inhibitor doxorubicin but not to vinblastine or ilmofosine (Table 1). These results illustrate that resistance to ilmofosine seems to be associated with multidrug resistance elicited by the P-glycoprotein. In order to substantiate this assumption, cells transfected with the MDR1-gene were used. Human HeLa cells transfected with a wild-type MDR1 gene (HeLa-MDR1-G185) or a Gly/Val mutation in position 185 (HeLa-MDR1-V185) are multidrug resistant and exhibit cross-resistance to ilmofosine, indicating that expression of the MDR1 gene is sufficient to cause cross-resistance to ilmofosine (Table 1). The extent of resistance to ilmofosine in MCF-7/ADR is similar to that to doxorubicin (resistance factor 7.13 and 8.7 respectively); in the other resistant cell lines tested, the resistance to ilmofosine is considerably lower than to vinblastine and doxorubicin (Table 1).

Combinations of ilmofosine with doxorubicin

The correlation between multidrug resistance and resistance to ilmofosine suggests that the phospholipid analogue may be a substrate for the MDR1-encoded P-glycoprotein. If this is the case, ilmofosine should act as a modulator of the P-glycoprotein activity. For this reason, we tried to reverse multidrug resistance. A combination of different concentrations of doxorubicin and ilmofosine in CCRF/CEM and CCRF/VCR1000 is shown in Figure 1A. No reversal of resistance could be observed. In addition, in the MDR1-transfected cell line (HeLa-MDR1-V185), also no reversal of doxorubicin resistance was achievable with ilmofosine (Figure 1B). The resistance of doxorubicin could also not be reversed by ilmofosine in CEM/VLB0 and MCF7/ADR cells.
which duplicated determinations are indicated in Materials and methods. The mean of three independent experiments, in which duplicate determinations were taken within each experiment (± s.e.m.), is indicated.

Figure 6  Effect of ilmosofine on cell multiplication of sensitive and resistant Meth A cells. Cell multiplication was determined after 72 h continuous incubation with the indicated concentrations of ilmosofine as described in Methods. The mean of three independent experiments, in which duplicate determinations were taken within each experiment (± s.e.m.), is indicated.

Figure 7  Inhibition of cell proliferation of MS and MR cells by vinblastine. The cells were continuously exposed to vinblastine for 72 h and counted by an electronic counter. Mean values (± s.e.m.) of two independent experiments (duplicate determinations within each assay) are indicated.

(data not shown). Ilmosofine was also not able to reverse the resistance to vinblastine in CCRF/VCR1000, HeLa-MDR1, CEM/VLB100 and MCF7/DOX cells (data not shown).

**Combination of ilmosofine with the multidrug resistance-reversing agent dexniguldipine-HCl**

If cross-resistance of multidrug-resistant cells to ilmosofine is caused by an increased export of ilmosofine by P-glycoprotein, the resistance to the compound should be reversible by a MDR-modulating agent. It has been shown previously that dexniguldipine-HCl (B8509-035) is approximately ten times more potent in reversing multidrug resistance than verapamil. To reverse multidrug resistance completely, 0.1 μM of the compound is sufficient (Hofmann et al., 1992). In order to investigate whether inhibition of P-glycoprotein by dexniguldipine-HCl increases the antiproliferative activity of ilmosofine, we treated CCRF/VCR1000 cells with both drugs concomitantly. Figure 2 shows the antiproliferative effects of ilmosofine (5–75 μM), dexniguldipine-HCl (0.5–7.5 μM) and a combination of both drugs (constant ratio 10:1). Dexniguldipine-HCl is not able to enhance the antiproliferative activity of ilmosofine in multidrug-resistant CCRF/VCR1000 cells at any of the concentrations applied, indicating again that ilmosofine seems not to be a substrate of P-glycoprotein.

**Effects of ilmosofine on P-glycoprotein**

In order to substantiate the conclusion that the resistance of MDRI-overexpressing cells to ilmosofine is not due to an enhanced P-glycoprotein-catalysed efflux of the phospholipid analogue, the interaction of ilmosofine with the P-glycoprotein was investigated. The fluorescent dye rhodamine 123 has been shown to act as an excellent substrate for the P-glycoprotein (Hofmann et al., 1992). Figure 3 demonstrates that dexniguldipine-HCl increases the intracellular level of rhodamine 123 by inhibiting the P-glycoprotein mediated efflux. The level of intracellular rhodamine after treatment with 1 μM dexniguldipine-HCl...
is in the range of sensitive cells (Hofmann et al, 1992). In contrast, ilmofosine does not exhibit a significant effect on rhodamine accumulation (Figure 3). A combination of ilmofosine with dexniguldipine-HCl enhances the effect of dexniguldipine on rhodamine accumulation (Figure 3). This result seems to indicate an enhanced accessibility of P-glycoprotein to the dexniguldipine action (and not competition for the binding site), probably because of an increased membrane fluidity under the influence of ilmofosine. The data also demonstrate that the MDR-modulating potency of dexniguldipine-HCl is not reduced in the presence of a tenfold molar excess of the phospholipid analogue. On the contrary, there is a potentiation of the inhibitory effect of dexniguldipine-HCI.

That ilmofosine indeed enhances the accessibility of P-glycoprotein ligands is also evident from photoaffinity studies. The dihydropyridine azidopine is a well-established photoaffinity label for P-glycoprotein. Figure 4 demonstrates that there is no competition between ilmofosine and [3H]azidopine. On the contrary, ilmofosine in this experiment also renders P-glycoprotein more accessible and increases the binding of azidopine. Furthermore, no significant alteration of the P-glycoprotein ATPase activity by ilmofosine could be detected, also indicating no direct interaction of ilmofosine with P-glycoprotein (Figure 5).

Resistance of Meth A cells

As described above, multidrug-resistant cells are cross-resistant to ilmofosine. The question was whether cells selected for resistance to ilmofosine are multidrug resistant and therefore cross-resistant to drugs transported by P-glycoprotein. In order to investigate this question, we used murine Meth A fibrosarcoma cells sensitive and resistant to ilmofosine. The resistance was obtained by addition of increasing concentrations of the phospholipid analogue ET-18-OCH₃ (1-octadecyl-2-O-rac-glyero-3-phosphocholine) (Petersen et al, 1992). The cells are cross-resistant to ilmofosine and have been grown in the presence of 6 μg ml⁻¹ (equivalent to 11.7 μM) ilmofosine for long periods of time. Dose–response curves of the sensitive and resistant cell lines are shown in Figure 6. Both cell lines exhibit similar sensitivity to vinblastine (Figure 7), indicating that long-term treatment with ilmofosine does not lead to a multidrug-resistant phenotype. Also short-term treatment with the compound does not increase the MDR1 mRNA levels, as shown in Figure 8.

Expression of the MDR2 gene

It has been reported recently that the MDR2-encoded P-glycoprotein transports phosphatidylcholine out of the cell (Smit et al, 1993; Ruetz et al, 1994). For this reason, we investigated whether this gene is involved in the resistance to ilmofosine by detection of the MDR1 and MDR2 mRNA levels. In Figure 9, the amount of MDR1 mRNA and that of the MDR2 mRNA in the different cell lines is shown. In sensitive HeLa-WT and resistant MDR1-overexpressing HeLa cells, no MDR2 expression was observed. Both MCF-7 cell lines exhibit similar levels of MDR2 mRNA (Figure 9). These results argue against a role of MDR2 in the resistance to ilmofosine.

DISCUSSION

Our results show that the MDR1-overexpressing cells MCF-7/ADR, CEM/VLB₅₀₀, CCRF/VCR1000, CCRF/ADR5000 and two HeLa cell lines transfected with MDR1 genes are cross-resistant to ilmofosine. Although the resistance to ilmofosine is considerably lower than to vinblastine or doxorubicin, a three- to sevenfold resistance can cause treatment failure in patients. Altered topoisomerase II-resistant CEM/VM-1 cells do not exhibit cross-resistance to the compound. The data obtained with HeLa-MDR1-G185 and HeLa-MDR1-V185 cells indicate that elevation of the expression of MDR1 by transfection of the gene is sufficient to elicit cross-resistance to ilmofosine and that no additional mechanism seems to be involved. P-glycoprotein transports lipophilic compounds out of the cell. Therefore, cross-resistance of multidrug-resistant cells could be caused by increased efflux of ilmofosine catalysed by the P-glycoprotein. Our data argue against this possibility: (1) ilmofosine does not enhance the antiproliferative activity of doxorubicin (Figure 1A and B) or vinblastine (data not shown); (2) the potent P-glycoprotein modulator dexniguldipine-HCl is not able to affect the sensitivity to ilmofosine (Figure 2); (3) a tenfold excess of ilmofosine does not compete with the P-glycoprotein inhibitory effect of
dexigulpine-HCl, as shown by the intracellular accumulation of rhodamine 123 (Figure 3); (4) ilmofosine does not compete with azidopine for the binding site (Figure 4); and (5) the ATPase activity determined in presence of ilmofosine is not altered significantly (Figure 5). This is an indication that ilmofosine does not interact directly with the P-glycoprotein, a conclusion that has recently also been reported by Principe et al (1994). A possible explanation for cross-resistance of multidrug-resistant cells to ilmofosine is that P-glycoprotein-expressing cells have an altered lipid composition, which renders cells less susceptible to the antiproliferative activity of ilmofosine (Endicott and Ling, 1989). In addition, P-glycoprotein seems to be involved in the transport of sterols from the endoplasmic reticulum (Metherall et al, 1996); this may lead to altered lipid composition of membranes, leading to resistance to ilmofosine. It was reported previously that a unique glycosphingolipid pattern is associated with multidrug resistance in MCF7 cells (Lavie et al, 1996). Le Moyec et al (1996) showed by nuclear magnetic resonance spectroscopy that cellular lipids are involved in the MDR1-mediated resistance to doxorubicin and taxol. Our results are consistent with these reports. Resistance of MDR1/-expressing cells to ilmofosine seems not to be a direct but an indirect effect of P-glycoprotein action. MDR1 expression leads to lipid and membrane alterations, and these alterations seem to cause resistance to ilmofosine.

It was shown that phosphatidylcholine is transported by the MDR2-encoded P-glycoprotein (Smit et al, 1993; Ruetzet et al, 1994). Because ilmofosine is a phosphatidylcholine analogue, it could be speculated that MDR2 is responsible for differences in the sensitivity to the compound. Our results argue against this possibility (Figure 9). Thorgeirsson et al (1991) proposed a possible mechanism for co-induction of the MDR1 and MDR2 gene. We could not observe a co-expression of both genes in our cell lines (Figure 9).

Imlofosine is an inhibitor of protein kinase C (Hofmann et al, 1989). There is experimental evidence that phosphorylation of P-glycoprotein by protein kinase C modulates the activity of the efflux pump (Ma et al, 1991; Chambers et al, 1992; Ahmad and Glazer, 1993). For the protein kinase C inhibitor ilmofosine, this mechanism seems to be of minor importance because the compound does not reduce multidrug resistance (Figure 1A and B; data not shown). KB-8-5 cells are the only cells in which ilmofo-
sine is able to reverse multidrug resistance. These cells are also not cross-resistant to ilmofosine (data not shown). The reason may be that KB cells are exceptionally sensitive to phospholipid analogues (Fleer et al, 1993).

It has been reported that modulation of protein kinase C activity may alter the expression of the MDR1 gene (Chaudhary and Roninson, 1992; Sampson et al, 1993). Ilmofosine, however, does not influence the expression of the MDR1 gene as detected by poly-
merase chain reaction (Figure 8). Long-term exposure of cells to ilmofosine, as shown in MR cells that are grown in the presence of the drug, did not lead to a multidrug-resistant phenotype. As shown in Figure 7, there is no difference in sensitivity to vinblastine in MS and MR cells. From these experiments, it can be concluded that treatment of patients with ilmofosine may not induce multidrug resistance. Multidrug resistance in patients is usually in the range of MCF7/ADR cells or lower. As shown in Table 1, the resistance of the MCF7/ADR cell line to ilmofosine is in the same range as doxorubicin. Therefore, the question whether expression of the MDR phenotype in patients may influence the effects of ilmofosine treatment in the clinic has to be addressed.

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ABBREVIATIONS

Dexigulpine-HCl, B8509-035, (4R)-3-[4,4-diphenyl-1-piperidinyl(propyl)]-5-methyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylate-hydrochloride; EGTA, ethyleneglycol-bis(β-aminoethyl)ether N,N,N',N'-tetraacetic acid; HeLa-WT, drug-sensitive HeLa wild-type cells; HeLa-MDR1-G185, HeLa-WT cells transfected with a MDR1 wild-type gene containing a glycine in position 185; HeLa-MDR1-V185, HeLa-WT cells transfected with a mutant MDR1 gene containing a valine in position 185; ilmofosine, BM 41,440, 3-hexadecyl-mercaptopo-2-methoxyethylpropyl-1-phosphocholine; MDR, multidrug resistance; MDR1, multidrug resistance gene 1; MDR2, multidrug resistance gene 2; MS, Meth A cells sensitive to ilmofosine; MR, Meth A cells resistant to ilmofosine; PCR, polymerase chain reaction

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