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Collateral sensitivity of multidrug resistant cells to narcotic analgesics is due to effects on the plasma membrane

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Abstract

It has previously been demonstrated that opiates interact directly with P-glycoprotein in drug resistant Chinese hamster ovary (CHO) cells (Callaghan, R. and Riordan, J.R. (1993) J. Biol. Chem. 268, 16059–16064). In this study we have examined the effects of several opiates on the growth of drug sensitive and resistant CHO and human MCF7 cell lines. The growth of P-glycoprotein expressing cells was inhibited by the opiates pentazocine, pethidine and naloxone to a greater extent than in drug sensitive cells. Since P-glycoprotein is localised at the plasma membrane the effects of opiates on membrane biophysical properties were investigated. The opiates caused a fluidizing effect in membranes from P-glycoprotein expressing cells and decreased the basal level of P-glycoprotein phosphorylation. In addition, they were able to increase the leakage of the membrane impermeant compound 6-carboxyfluorescein entrapped in model membrane vesicles. The ability to alter membrane biophysical properties correlated with the inhibitory effects on growth of drug resistant cells. These results suggest that the collateral sensitivity of P-glycoprotein expressing cell lines to opiates is mediated by the drugs' effects on the plasma membrane.

Keywords: Multidrug resistance; Analgesic; Plasma membrane; CHO cell line; MCF7 cell line

1. Introduction

The phenomenon of multi-drug resistance has been widely investigated since it is a serious obstacle in the chemotherapeutic treatment of malignancies (reviewed in [1–3]). Resistant cells frequently overexpress a 180 kDa membrane glycoprotein, P-glycoprotein [4], which is widely believed to act as an ATP-dependant drug efflux pump [2]. The search for xenobiotics that reverse multi-drug resistance by competing with cytotoxic agents for transport by P-gp has been vigorous. Indeed several agents such as the calcium channel blockers [5], cyclosporin A [6], phenothiazines [7], anti-estrogens [8] and an antihistamine [9] have been shown to reverse multi-drug resistance. However, clinical trials with many reversing agents been limited by toxicities of these agents [10,11]. More novel strategies for reversal of the MDR phenotype in vitro have been based on biochemical differences between sensitive and resistant cells.

Several drug resistant cell lines display altered metabolism of high energy compounds such as ATP, ADP and phosphocreatine [12]. Furthermore, the antimetabolites sodium azide, deoxyglucose and lonidamine, an inhibitor of aerobic glycolysis, have been used to reverse MDR in vitro [13–15]. Another biochemical difference between drug sensitive and resistant cell lines is increased cytosolic pH in P-gp expressing cells [16]. MDR reversal agents such as verapamil are able to restore cytosolic pH levels to those in drug sensitive cells. Furthermore, it has recently been suggested that pH and/or ionic gradients across the plasma membrane of drug resistant cells directly generated by P-gp are responsible for the lower accumulation of cytotoxic drugs [17–19].

Certain growth conditions and substances have been demonstrated to be more toxic to MDR cells than drug sensitive parental lines ([20] for review). This phe-

Abbreviations: CHO, Chinese hamster ovary; P-gp, P-glycoprotein; MDR, multiple drug resistance; 6-CF, 6-carboxyfluorescein; PKC, protein kinase C.

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nomenon, called hyper- or collateral sensitivity, in drug resistant cells has been demonstrated for verapamil [21], local anaesthetics [22], detergents [23], and long chain unsaturated fatty acids [24]. Interestingly, several compounds which cause collateral sensitivity are not substrates for binding or transport by P-glycoprotein and a mechanism of collateral sensitivity has not yet been elucidated. In this study we report that drug resistant cells are collaterally sensitive to several of the opiate class of narcotic analgesics. Furthermore, a correlation between their potency in causing hypersensitivity in resistant cells and ability to alter membrane biophysical properties has been established.

2. Materials and methods

2.1. Chemicals

Morphine sulfate, naloxone hydrochloride, pethidine hydrochloride and pentazocine lactate were all purchased from The Hospital for Sick Children Pharmacy (Toronto, Ontario). [^{32}P]Orthophosphate was obtained from Amersham (Ontario, Canada). Verapamil hydrochloride, daunomycin and adriamycin were purchased from Sigma (St. Louis, MO). Phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine were purchased from Avanti Polar Lipids. 6-Carboxyfluorescein was purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained from regular suppliers and were of analytical grade.

2.2. Tissue culture

Drug sensitive Chinese hamster ovary cells (AuxB1) and the drug resistant line (B30) were grown in α-minimum essential medium supplemented with 10% heat inactivated bovine serum, streptomycin (100 ng/ml) and penicillin (100 ng/ml) as previously described [21]. The resistant cells were maintained in medium containing colchicine (30 μg/ml). The human breast carcinoma drug sensitive (MCF7) and drug resistant (MCF7^{Adr}) cell lines were a generous gift from Dr. Kenneth Cowan (NCI, Bethesda, MD). These cell lines were also grown in α-minimum essential medium supplemented with 10% heat inactivated bovine serum, streptomycin (100 ng/ml) and penicillin (100 ng/ml) [25]. At each third passage the MCF7^{Adr} cell line was maintained in 3.8 μg/ml adriamycin.

2.3. P-glycoprotein detection

The presence of P-glycoprotein in B1, B30, MCF7 and MCF7^{Adr} cells was determined by Western blot analysis based on published methods [26]. Total cell protein was extracted in 1% SDS and 20 μg loaded on 6% SDS-PAGE gels. A micro-Lowry assay, using bovine serum albumin as a standard, was used to quantitate protein concentration. Proteins were electrophoretically transferred onto a nitrocellulose filter and immunoblotting was performed using the anti-Pgp monoclonal antibody C219 (Centocor Diagnostics).

2.4. Cell cytotoxicity assay

Cytotoxicity assays were based on previously published methods [21]. Briefly, cells from all lines were seeded in 24-well tissue culture plates (Costar) at a density of 4000 cells per well. Cells were allowed to adhere for 24 h at which time medium was supplemented with opiates in the concentration range of 5–150 μM. The cells were left to grow for 7–10 days at which time medium was aspirated and cells washed four times with phosphate-buffered saline (PBS). Subsequently, the cells were fixed and stained for 30 min in 50% methanol containing 5 g/l methylene blue. Each well was extracted with 2 ml 1% (w/v) SDS and the absorbance read at 650 nm. Cell survival was expressed as a percentage of absorbance obtained in the absence of drug. The IC_{50} dose for each drug was defined as the concentration at which the compound caused a 50% decrease in cell growth.

2.5. Liposomes containing 6-carboxyfluorescein

Unilamellar liposomes composed of PC/PS/PE (3:4:3) were made as follows. A film of phospholipid (10 mg total lipid) was prepared in glass tubes using reverse phase evaporation. 6-Carboxyfluorescein (50 mM) was dissolved in 200 μl 10 mM Tris-HCl (pH 8.3) and the pH of the final solution was adjusted to 7.4 with 1 M HCl. 6-Carboxyfluorescein is a hydrophilic compound whose fluorescence is self-quenched at concentrations of 50 mM and above [27]. The 6-CF (50 mM) containing solution was used to hydrate the lipid film and the suspension was subjected to 10 rounds of rapid freeze-thawing. At this stage the solution is composed of multi-lamellar phospholipid vesicles encapsulating 6-CF. The multi-lamellar vesicles were then passed through a 200 nm nitrocellulose filter (20–25 cycles) in a commercial extruder (Avestin, Ottawa) to generate uniform size unilamellar vesicles encapsulating 6-CF. The liposome solution was subsequently passed through disposable PD-10 columns (Pharmacia, Uppsala) containing Sephadex G-25 media to remove unencapsulated 6-CF. Fractions (1 ml) were collected and those containing loaded liposomes kept on ice for not more than 8 h.

2.6. Effects of opiates on leakiness of model lipid bilayers

Loaded liposomes (20 μl) were added to 2 ml of buffer composed of 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. Fluorescence spectra of 6-carboxyfluorescein were recorded in a Hitachi 2000 fluorimeter with instrument

settings as follows; temperature 37° C, excitation wavelength 493 nm, emission wavelength 535 nm and both slit widths at 3 nm. Samples were stirred continuously and the reaction begun with the addition of drug. Total reaction time was 60 min at which point 50 μl of 10% (w/v) Triton X-100 was added to disperse the liposomes and allow determination of total amount of fluorescence associated with the encapsulated 6-CF. Results were plotted as the percentage of total fluorescence observed as a function of reaction time. The rate of leakage was determined from the initial slope of the fluorescence vs. time curves. The parameter EXT_{60} is defined as the amount of total fluorophore leaked from liposomes in the course of a 60 min incubation.

2.7. Phosphorylation of P-gp in B30 cells

The phosphorylation state of P-gp in B30 cells was determined using previously published methods [28]. Briefly, confluent monolayers of B30 cells (60 × 35 mm) were incubated with 300 μCi of [^{32}P]orthophosphate for 4 h at 37° C in the presence of 100 μM appropriate drug. Following the incubation period, cells were washed with PBS and harvested with 1 ml RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM Tris-HCl pH 7.2). The cell extracts were incubated with anti-P-gp antibody for 15 h and subsequently for 2 h with protein A Sepharose as described [26]. Immobilised protein was eluted with 20 μl Laemmli sample buffer at 37° C for 15 min and subjected to SDS-PAGE electrophoresis using 8% gels (Novex Systems). Gels were dried and proteins visualised by autoradiography.

2.8. Preparation of plasma membranes

Plasma membranes from B1 and B30 cells were prepared as previously described [29]. Membrane preparations were stored in 0.01 M Tris-HCl, pH 7.4, 0.25 M sucrose at −70° C prior to use. Protein concentration was determined using a micro Lowry method.

2.9. Lipid structural order in plasma membranes from B1 and B30 cells

The effects of opiates on lipid structural order in plasma membranes from B1 and B30 cells was determined by the electron paramagnetic resonance (EPR) spectra of 5-doxyl-stearic acid (5-SASL). Membranes (350 μg) were pelleted (150000 × g) using an Airfuge (Beckman Instruments), washed in buffer and aliquoted (50 μg) into 25 μl sucrose buffer containing 5-SASL and drug. The membrane-drug mixtures were left to stand for 20 min at room temperature, centrifuged as above, and washed five times to remove unincorporated probe. The mixture was then drawn into 50 μl capacity capillary tubes which were subsequently flame sealed. Membranes were pelleted by centrifugation at 5000 × g for 5 min and spectra recorded on a Varian ESR machine. Spectral parameters were as follows; scan range 100 Gauss, field set 3175.4 Gauss, modulation amplitude 1.6 Gauss, scan time 4 min at ambient temperature. Lipid structural order of plasma membranes in the presence/absence of drug was calculated from the EPR spectra of 5-SASL as previously described [30].

3. Results

3.1. Characterization of B1, B30, MCF7 and MCF7^{Adr} cell lines

Western blot analysis was used to demonstrate the levels of P-gp expressed in each cell line (Fig. 1). As expected no P-gp was detected in either of the drug sensitive parental lines, B1 and MCF7 (lanes 1 and 3). In contrast, both drug resistant cell lines (B30 and MCF7^{Adr}) expressed P-gp at considerable levels (lanes 2 and 4). The amount expressed, per mg of total cell protein, was considerably higher in B30 cells. Cytotoxicity assays were performed to examine the relative levels of resistance to colchicine in the B30 and MCF7^{Adr} cell lines. The IC_{50} dose of colchicine in B1 cells was 33 ± 16 nM as compared with 26.8 ± 3.4 μM in B30 cells. This represents an 820-fold increase in resistance to colchicine. In drug sensitive MCF7 cells the IC_{50} dose of colchicine was 1.9 ± 0.9 nM compared to 423 ± 48 nM in the resistant MCF7^{Adr} cell lines. This difference represents a 220-fold resistance to colchicine in MCF7^{Adr} cells. Therefore, increased resistance of B30 compared to MCF7^{Adr} cells was in qualitative agreement with the higher expression of P-gp based on Western blot analysis.

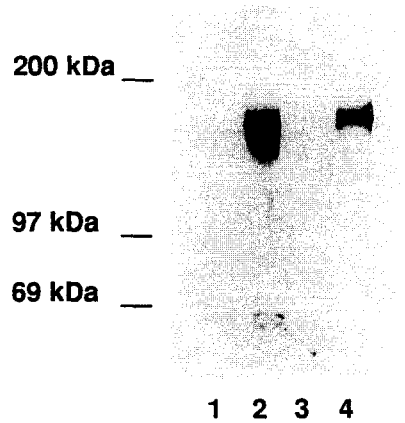

Fig. 1. Western blot analysis of P-gp expression in B1 (lane 1), B30 (lane 2), MCF7 (lane 3) and MCF7^{Adr} (lane 4) cell lines. All lanes were loaded with 20 μg cell protein and the blot probed with the monoclonal antibody C219.

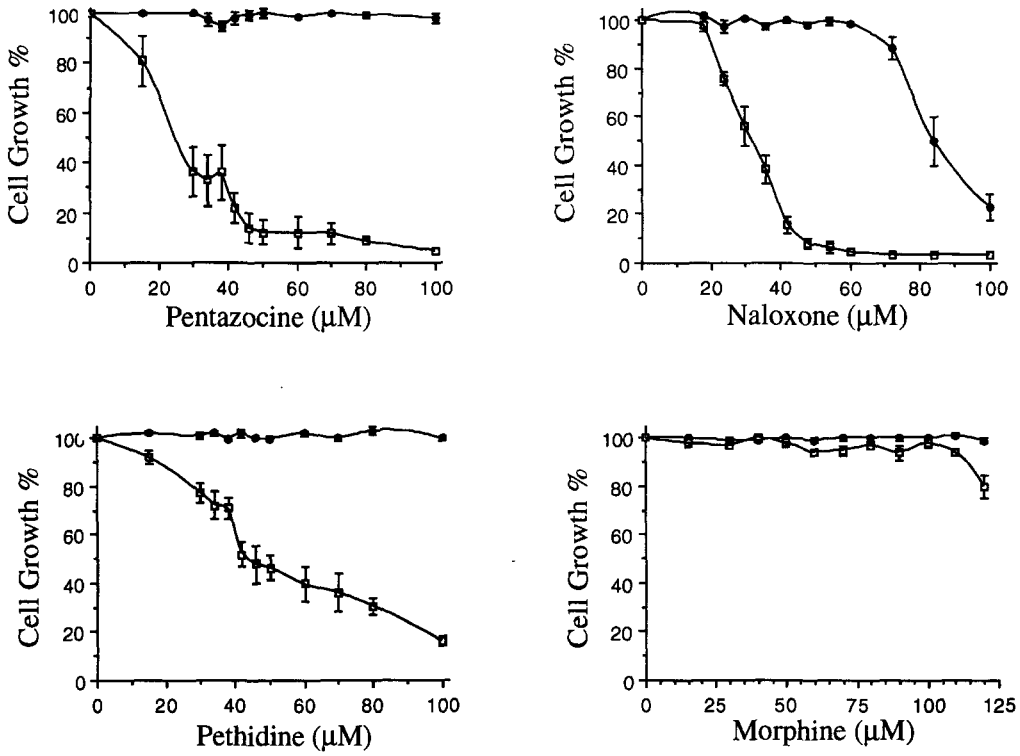

Fig. 2. Growth inhibitory effects of pentazocine (top left), naloxone (top right), pethidine (bottom left) and morphine (bottom right) on B1 (●) and B30 (□) cell lines. Cells were seeded at approx. 4000 per well at the drug concentration indicated. Results shown are mean (±S.E.) of three independent experiments.

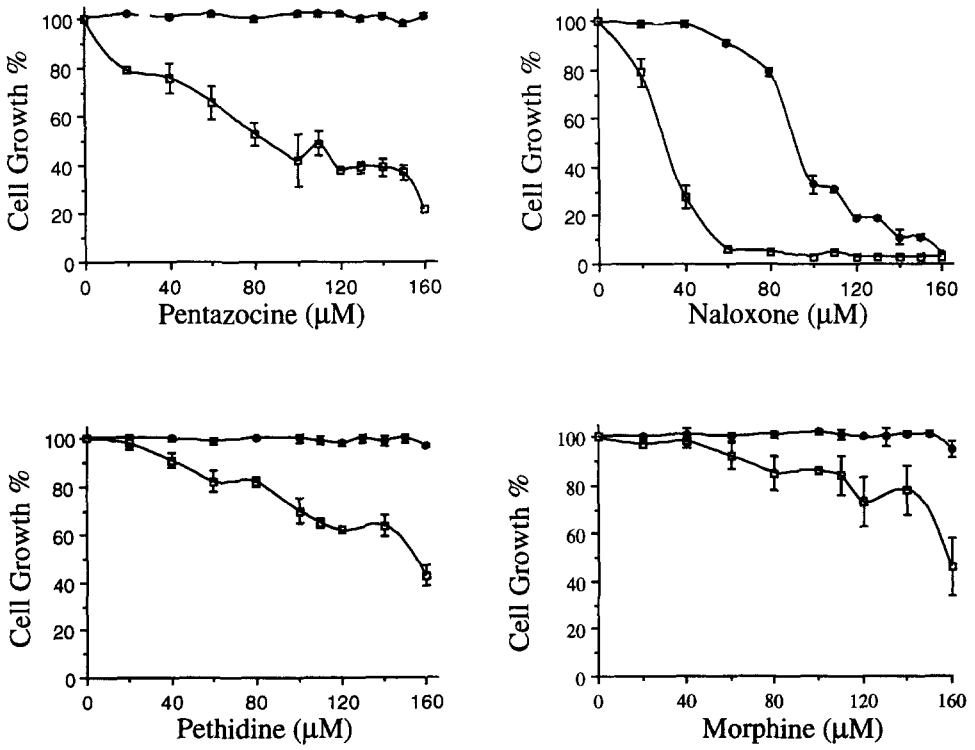

Fig. 3. Growth inhibitory effects of pentazocine (top left), naloxone (top right), pethidine (bottom left) and morphine (bottom right) on MCF7 (●) and MCF7^{Adr} (□) cell lines. Cells were seeded at approx. 4000 per well at drug concentration indicated. Results shown are mean (±S.E.) of three independent experiments.

3.2. Effects of opiates on growth of B1, B30, MCF7 and MCF7^{Adr} cell lines

The effects of naloxone, pentazocine, pethidine and morphine on the growth of drug sensitive (B1) and drug resistant (B30) cell lines is shown in Fig. 2. Naloxone caused a 50% reduction in growth of B1 cells at a concentration of 85 μM (IC_{50}). However, in B30 cells the IC_{50} for naloxone was 27 μM which is a 3.2-fold increase in sensitivity. Neither pentazocine nor pethidine caused a reduction in growth of drug sensitive B1 cells at concentrations up to 100 μM. In contrast, both drugs caused significant reductions in growth of drug resistant B30 cells. The respective IC_{50} values for pentazocine and pethidine were 22 μM and 42 μM which indicate significant hypersensitivity of B30 cells to these drugs. However, morphine did not have any effects on growth of B1 or B30 cells at concentrations up to 120 μM. Thus, the narcotic agonists pentazocine, pethidine and the narcotic antagonist naloxone, were able to cause marked reductions in the growth of drug resistant B30 cells compared to parental cells.

The above effects were observed in CHO cell lines which highly overexpress P-glycoprotein. As described above, MCF7^{Adr} cells have significantly less P-gp and are less resistant to colchicine than B30 cells. Therefore, the experiments on cell growth were repeated in drug sensitive and resistant human breast cancer cell lines to determine if the collateral sensitivity to opiates was dependant on P-gp levels and degree of resistance. The effects of opiates on cell growth of drug sensitive and resistant cell lines are shown in Fig. 3. Morphine did not affect the growth of the drug sensitive MCF7 cell line but impaired the growth of drug resistant MCF7^{Adr} cells. However, the growth inhibitory effects on MCF7^{Adr} cells was not large and the effects only occurred at concentrations of morphine greater then approx. 100 μM. Naloxone was the only compound found to inhibit the growth of drug sensitive MCF7 cells. The IC_{50} value for naloxone is 90 μM which is similar to effects on drug sensitive B1 cells. Pentazocine and pethidine did not cause any growth inhibitory effects in MCF7 cells at concentrations up to 150 μM. However, drug resistant MCF7^{Adr} cells remained hypersensitive to pentazocine, pethidine and naloxone. The respective IC_{50} values were 80 μM, 120 μM and 30 μM. Thus the narcotic agonists pethidine, pentazocine and the narcotic antagonist naloxone were also able to inhibit preferentially, the growth of drug resistant MCF7^{Adr} cells. However, the IC_{50} concentrations were higher than observed in drug resistant B30 cells. Therefore, it appears that the susceptibility of drug resistant cell lines to the growth inhibitory effects of opiates correlates with the degree of resistance and amount of P-gp expressed in the respective cell lines (see Fig. 2).

Table 1
The effects of morphine, naloxone, pethidine, pentazocine and verapamil on lipid structural order in plasma membranes from drug sensitive (B1) and drug resistant (B30) cells

Compound	B1	B30
Control	0.495 ± 0.003	0.501 ± 0.007
Morphine	0.474 ± 0.021	0.485 ± 0.001
Naloxone	0.476 ± 0.003 [a]	0.488 ± 0.007
Pethidine	0.487 ± 0.010	0.487 ± 0.015
Pentazocine	0.482 ± 0.018	0.478 ± 0.004 [a]
Verapamil	0.496 ± 0.009	0.465 ± 0.007 [a]

All values were obtained from three independent experiments and are expressed as mean ± S.E.
[a] Statistically significant difference from the control value in the same cell line ($P < 0.05$).

3.3. Opiate induced alterations in lipid structural order in B1 and B30 plasma membranes

Lipid structural order was investigated using the lipophilic probe 5-SASL which has previously been demonstrated to localise at the plasma membrane [31]. Table 1 shows the effects of opiates and verapamil on the structural order of 5-doxyl-stearic acid in plasma membranes from drug sensitive and resistant CHO cells. Verapamil was included since it has been previously established as a potent collateral sensitizing agent in B30 cells [32]. There was no significant difference in gross plasma membrane lipid structural order between B1 and B30 cells which is in agreement with previous reports [20]. Verapamil and opiate analgesics were unable to alter structural order to statistically significant levels in drug sensitive cells. Naloxone, an opiate antagonist reduced structural order in plasma membranes from B1 cells and was the only compound tested to cause significant inhibition of drug sensitive cell growth. Verapamil and the opiate pentazocine both caused statistically significant reductions in lipid structural order in plasma membranes from B30 cells. These two compounds are potent agents in causing collateral sensitivity of drug resistant B30 cells. A decrease in lipid structural order, also referred to as membrane fluidity, indicates increased motion by the acyl chains of membrane lipids [33]. Naloxone and pethidine caused smaller reductions in lipid structural order, however, the reductions did not reach statistical significance. Nonetheless, there appears to be a correlation between potency in causing growth inhibition of drug resistant cells and effects of drugs on plasma membrane lipid structural order. These results may suggest that the expression of P-gp renders membranes more susceptible to gross changes in fluidity when exposed to verapamil and some opiate analogues.

3.4. Leakiness of PC / PS / PE unilamellar vesicles

The ability of opiates to induce leakage of the hydrophillic fluorescent dye 6-CF trapped inside unilamellar vesicles is shown in Fig. 4. The effect was immediate and had not reached a plateau at 60 min after addition of drug. The initial rate and the extent of leakage after 60 min (EXT_{60}), from liposomes was determined for each drug at

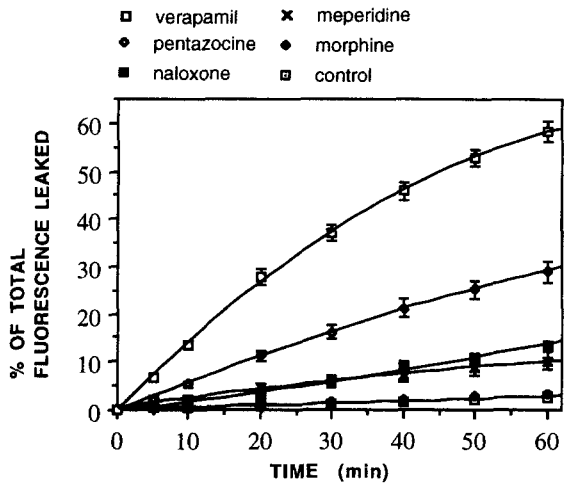

Fig. 4. Effects of several compounds on the leakage of 6-CF entrapped in unilamellar vesicles composed of PC/PS/PE (3:4:3). Values plotted expressed as the % of total fluorescence observed as a function of reaction time. All drug concentrations were 0.4 mM. Values are the mean (±S.E.) obtained from three independent experiments. (Meperidine is a synonym for pethidine.)

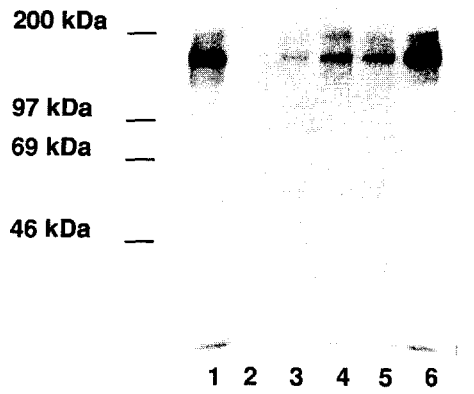

Fig. 5. The effects of several drugs on the phosphorylation state of P-gp in drug resistant B30 cells. Cells were labelled with [^{32}P]orthophosphate for 4 hours as described in Materials and methods. P-gp has a basal state of phosphorylation (lane 1). The effects of several compounds on the basal level of phosphorylation was examined: (lane 2) naloxone, (lane 3) morphine, (lane 4) pentazocine, (lane 5) pethidine and (lane 6) verapamil.

three concentrations and the data summarized in Table 2. In the absence of any drug the rate of 6-CF leakage from liposomes was almost negligible with less than 3% of total entrapped 6-CF leaking after 60 min incubation. Verapamil, pentazocine, pethidine and naloxone, were able to significantly increase both the rate and EXT_{60} above the levels obtained in the absence of drug at all concentrations examined. The increases in rate and EXT_{60} occurred in a dose-dependant manner with verapamil and pentazocine

Table 2
The effect of opiates on the leakage of 6-carboxyfluorescein from PC/PS/PE (3:4:3) unilamellar liposomes at 37° C

	Initial rate (%/min)	EXT_{60} (% total)
Control	0.048±0.010	2.7±0.4
Morphine		
100 μM	0.039±0.006	3.4±0.3
200 μM	0.055±0.011	3.7±0.2
400 μM	0.070±0.005	3.5±0.1
Pethidine		
100 μM	0.131±0.023 [a]	6.9±0.8 [a]
200 μM	0.189±0.024 [a]	8.7±1.0 [a]
400 μM	0.215±0.043 [a]	10.2±1.9 [a]
Naloxone		
100 μM	0.130±0.030 [a]	4.4±1.6
200 μM	0.160±0.050 [a]	7.8±3.2 [a]
400 μM	0.320±0.050 [a]	13.3±0.4 [a]
Pentazocine		
100 μM	0.177±0.026 [a]	9.1±1.2 [a]
200 μM	0.314±0.040 [a]	15.3±1.7 [a]
400 μM	0.634±0.051 [a]	29.1±2.3 [a]
Verapamil		
100 μM	0.260±0.010 [a]	12.9±1.2 [a]
200 μM	0.620±0.040 [a]	24.0±1.6 [a]
400 μM	1.520±0.120 [a]	58.3±2.2 [a]

Excitation wavelength 493 nm and emission wavelength 535 nm.
[a] Values significantly different from control data ($P < 0.05$).

the most potent permeabilizing agents, leading to increases in the initial rate of 6-CF leakage by 5.4 and 3.7-fold respectively at 100 μM concentrations. These two compounds also caused the most marked changes in drug resistant plasma membrane fluidity and pentazocine was the most potent opiate collateral sensitizer. Pethidine and naloxone exhibited intermediate potency in 6-CF leakage while morphine did not significantly affect permeability at any of the concentrations examined. Morphine also did not significantly alter membrane fluidity or confer collateral sensitivity to B30 cells. Ability to induce leakage of the hydrophilic compound 6-CF entrapped in model liposomes represents significant alteration of membrane biophysical properties. The order of potency in causing leakage of 6-CF from model lipids by opiates correlates well with the respective ability of these drugs to confer collateral sensitivity in resistant B30 and $MCF7^{Adr}$ cells.

3.5. Modification of P-gp phosphorylation by opiates

Agents which confer collateral sensitivity have been demonstrated to increase the basal phosphorylation state of P-gp [34]. Drug resistant B30 cells were labelled with [^{32}P]orthophosphate in the presence or absence of drug for 4 h and P-gp was subsequently immunoprecipitated and subjected to electrophoresis. The results are shown in Fig. 5. In the control lane a band corresponding to P-gp was observed at 180 kDa. This basal level of P-gp phosphorylation has previously been demonstrated [28]. Incubation with verapamil did not significantly alter the phosphorylation state of P-gp, which is in contrast to the effects seen with opiates. All opiates tested were able to significantly reduce the phosphorylation of P-gp and the order of potency was naloxone > morphine > pentazocine > pethidine as determined by densitometric analysis. Whole cell lysates

following metabolic labelling with [^{32}P]orthophosphate were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. There was no significant effect of any opiate on total protein phosphorylation state (data not shown). This indicates that the effects of opiates on phosphorylation were not due to non-specific effects such as ATP-depletion. The ability of opiates to alter the phosphorylation state of P-gp did not, however, correlate precisely with the growth inhibition potency shown in Fig. 2. Alteration of membrane biophysical properties has been shown to modify the activity of protein kinase C, which is known to phosphorylate P-gp [35].

4. Discussion

The phenomenon of collateral or hypersensitivity was first described by Bech-Hansen in 1976 [23] and since then several compounds have been shown to cause growth inhibition of drug resistant cells [36]. In the present study we have demonstrated that several opiate analogues were able to inhibit the growth of drug resistant cells at much lower doses than in drug sensitive parental lines. Opiates have also recently been shown to inhibit the binding and transport of vinca alkaloids by P-glycoprotein in drug resistant cells [37]. Verapamil, which is a very strong inducer of collateral sensitivity [32], also inhibits cytotoxic drug binding to P-gp [5]. The growth inhibition of drug resistant cells to opiates in the present study appeared to be a function of the relative resistance and amount of P-gp present. A similar relationship between level resistance to cytotoxicity and hypersensitivity to a series of detergent compounds has been described [23]. Therefore, it appears that P-gp expression has a major role in rendering cells vulnerable to collateral sensitizing agents.

However, many collateral sensitivity agents such as local anaesthetics, detergents and fatty acids do not appear to act directly on P-gp [36]. The common property shared by collateral sensitizers is their ability to alter membrane biophysical properties [22–24,36] suggesting that toxicity is mediated at the plasma membrane level. Several reports have described a number of alterations to plasma membrane biophysical properties in resistant cells compared to parental cells [20,38]. Specifically, increased fragility to shear forces, elevated rates of endocytosis, a decreased surface hydrophobicity and an increased intramembranous particle density have been described in various drug resistant cell lines [38–40]. The absence of gross differences in lipid composition between drug resistant and sensitive cells suggests that expression of P-gp is responsible for the altered membrane properties [38,39]. In turn, several compounds and growth conditions which alter membrane biophysical properties are able to reverse MDR and/or confer collateral sensitivity [24,38,41].

In this study the potent collateral sensitizer verapamil and several of the opiates altered membrane biophysical properties such as increased membrane fluidity to varying degrees in drug resistant cells. Bulk phase fluidity describes the motion of membrane phospholipid acyl chains [33] and alterations in plasma membrane fluidity have previously been shown to alter P-gp dependant drug transport [41,42]. Verapamil and the opiates investigated in this study are hydrophobic molecules that would be expected to cause disruption of phospholipid movement upon intercalation into lipid bilayers. The increased alteration of fluidity in drug resistant cell membranes compared to membranes from parental cells may reflect the inherent instability of bilayers containing P-gp [38–40]. Modification of membrane lipid structural order, or fluidity, may also lead to altered permeability properties of plasma membranes. Indeed, opiates and verapamil were shown to affect the lipid bilayer of model liposomes sufficiently to allow passage of an entrapped polar fluorescent dye, 6-CF. Several drugs and hydrophobic compounds which disrupt phospholipid packing in bilayers have also been demonstrated to increase membrane permeability [43,48]. In the present study, the ability of opiates to increase membrane permeability to 6-CF correlated well with the drugs collateral sensitization potency. Furthermore, other collateral sensitizing agents have been shown to increase membrane permeability in non-Pgp expressing cells [21] suggesting an effect on the membrane lipid phase. As mentioned earlier several reports have indicated that the expression of P-gp leads to severe alterations in plasma membrane biophysical properties [38–40] resulting in a more unstable or less rigid membrane. Consequently, further disruption of plasma membrane biophysical properties such as fluidity and permeability by opiates may have consequences for drug resistant cell viability.

Opiates were also shown to reduce the basal phosphorylation state of P-gp. Phosphorylation of P-gp has been shown to control specificity and influence the activity of drug transport [47]. It has also been demonstrated that administration of trifluoperazine and *N*-ethylmaleimide lead to hyperphosphorylation of P-gp in drug resistant cells [34]. In the same study it was postulated that the hyperphosphorylation of P-gp by trifluoperazine and *N*-ethylmaleimide resulted in collateral sensitivity of drug resistant cells to these agents [34]. P-gp is phosphorylated by protein kinase C [44,45] whose activity is controlled by calcium and membrane biophysical properties [46]. Several compounds which affect membrane biophysical properties have been shown to alter PKC activity ([46] for review). In the present study although we have not shown a direct correlation between changes in P-gp phosphorylation and collateral sensitivity, a link cannot be entirely ruled out. What is clear is that opiates are able to modify membrane biophysical properties. This alteration in membrane characteristics may modify the activity of PKC, an enzyme playing a major role in phosphorylation of P-gp. However, the consequences of decreased P-gp phosphorylation by opiates for cell viability is not clear.

In summary, the present study demonstrates that opiates induce collateral sensitivity in drug resistant cells expressing P-gp. The degree of hypersensitivity appears to be proportional to the amount of P-gp and the mechanism related to the membrane perturbing properties of opiates. Several investigations indicate that the membranes of drug resistant cells are less stable. Therefore, the membrane perturbing effects of opiates and verapamil may preferentially target drug resistant cells, thus compromising cell integrity and consequently cell viability.

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References

[1] Riordan, J.R. and Ling, V. (1985) Pharmac. Ther. 28, 51–75.
[2] Endicott, J.A. and Ling, V. (1989) Annu. Rev. Biochem. 58, 137–171.
[3] Bellamy, W.T., Dalton, W.S. and Dorr R.T. (1990) Cancer Invest. 8, 547–562.
[4] Kartner, N., Riordan, J.R. and Ling V. (1983) Science 221, 1285–1288.
[5] Cornwell, M.M., Gottesman, M.M. and Pastan, I. (1987) J. Biol. Chem. 262, 2166–2170.
[6] Ryffel, B., Woerly, G., Rodriguez, C. and Foxwell, B.M.J. (1991) J. Rec. Res. 11, 675–686.
[7] Ford, J.M., Prozialeck, W.C. and Hait, W.N. (1988) Mol. Pharm. 35, 105–115.
[8] Kirk, J., Houlbrooks, S., Stuart, N.S.A., Stratford, I.J., Harris, A.L. and Carmichael, J. (1993) Br. J. Cancer 67, 1189–1195.
[9] Hait, W.N., Gesmonde, J.F., Murren, J.R., Yang, J-M., Chen, H-X. and Reiss, M. (1993) Biochem. Pharm. 45, 401–406.
[10] Dalton, W.S., Grogan, T.M., Meltzer, P.S., Scheper, R.J., Durie, B.G., Taylor, C.W., Miller, T.P. and Salmon, S.E. (1989) J. Clin. Oncol. 7, 415–424.
[11] Durie, B.G.M. and Dalton, W.S. (1988) Br. J. Haematol. 68, 203–206.
[12] Kaplan, O., Navon, G., Lyon, R.C., Faustino, P.J., Straka, E.J. and Cohen, J.S. (1990) Cancer Res. 50, 544–551.
[13] Lyon, R.C., Cohen, J.C., Faustino, P.J., Megnin, F. and Myers, C.E. (1988) Cancer Res. 48, 870–877.
[14] Citro, G., Cucco, C., Verdina, A. and Zupi, G. (1991) Br. J. Cancer 64, 534–536.
[15] Versantvoort, C.H.M., Broxterman, H.J., Pinedo, H.M., De Vries, E.G.E., Feller, N., Kuiper, C.M. and Lankelma, J. (1992) J. Cancer Res. 52, 17–23.
[16] Thiebaut, F., Currier, S.J., Whitaker, J., Haughland, R.P., Gottesman, M.M., Pastan, I. and Willingham, M.C. (1990) J. Histochem. Cytochem. 38, 685–690.
[17] Altenberg, G.A., Young, G., Horton, J.K., Glass, D., Belli, J. and Reuss, L. (1993) Proc. Natl. Acad. Sci. USA 90, 9735–9738.
[18] Keizer, H.G. and Joenje, H. (1989) J. Natl. Cancer Inst. 81, 706–709.
[19] Roepe, P.D., Wei, L.-Y., Cruz, J. and Carlson, D. (1993) Biochemistry 32, 11042–11056.
[20] Alon, N., Busche, R., Tummler, B. and Riordan, J.R. (1991) In Molecular and Cellular Biology of Multi-Drug Resistance in Tumor Cells (Roninson, I., ed.), pp. 263–276.
[21] Cano-Gauci, D.F. and Riordan, J.R. (1987) Biochem. Pharm. 36, 2115–2123.
[22] Carlsen, S.A., Till, J.E. and Ling, V. (1976) Biochim. Biophys. Acta 455, 900–912.
[23] Bech-Hansen, N.T., Till, J.E. and Ling, V. (1976) J. Cell. Physiol. 88, 23–32.
[24] Zulstra, J.G., De Vries, E.G.E., Muskiet, F.A.J., Martini, I.A., Timmer-Bosscha, H. and Mulder, N.H. (1987) Int. J. Cancer 40, 850–856.
[25] Fairchild, C.R., Ivy, S.P., Kao-Shan, C.-S., Whang-Peng, J., Rosen, N., Israel, M.A., Melera, P.W., Cowan, K.H. and Goldsmith, M.E. (1987) Cancer Res. 47, 5141–5148.
[26] Chang, X-B., Tabcharani, J.A., Huo, Y-X., Jensen, T.J., Kartner, N., Alon, N., Hanrahan, J.W. and Riordan J.R. (1993) J. Biol. Chem. 268, 11304–11311.
[27] Weinstein, J.N., Yoshikami, J.D., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Science 195, 489–492.
[28] Fine, R.L., Pastel, J. and Chabner, B.A. (1988) Proc. Natl. Acad. Sci. USA 85, 582–586.
[29] Cornwell, M.M., Gottesman, M.M. and Pastan, I. (1986) J. Biol. Chem. 261, 7921–7928.
[30] Fraser, O.M., Louro, S.R.W., Horvath, L.I., Miller, K.W. and Watts, A. (1990) Biochemistry 29, 2664–2669.
[31] Siegfried, J.A., Kennedy, K.A., Sartorelli, A.C. and Tritton, T.R. (1983) J. Biol. Chem. 258, 339–343.
[32] Warr, J.R., Brewer, F., Anderson, M. and Ferguson, J. (1986) Cell Biol. Int. Rep. 10, 389–399.
[33] Campisi, J. and Scandella, C.J. (1980) J Biol. Chem. 255, 5411–5419.
[34] Center, M.S. (1985) Biochem. Pharm. 34, 1471–1476.
[35] Chambers, T.C., Chalikonda, I. and Eilon, G. (1990) Biochem. Biophys. Res. Commun. 169, 253–259.
[36] Cano-Gauci, D.F and Riordan, J.R. (1991) In Molecular and Cellular Biology of Multi-Drug Resistance in Tumor Cells (Roninson, I., ed.), pp. 337–347.
[37] Callaghan, R. and Riordan, J.R. (1993) J. Biol. Chem. 268, 16059–16064.
[38] Callaghan, R., Van Gorkom, L.C.M. and Epand, R.M. (1992) Br. J. Cancer 66, 781–786.
[39] Riordan, J.R. and Ling, V. (1979) J. Biol. Chem. 254, 12701–12705.
[40] Arsenault, L.A., Ling, V. and Kartner, N. (1988) Biochim. Biophys. Acta 938, 315–321.
[41] Callaghan R., Stafford, A. and Epand, R.M. (1993) Biochim. Biophys. Acta 1175, 277–282.
[42] Sinicrope, F.A., Dudeja, P.K., Bissonnette, B.M., Safa, A.R. and Brasitus, T.A. (1992) J. Biol. Chem. 267, 24995–25002.
[43] In 't Veld, G., Driessen, A.J.M. and Konings, W.N. (1992) Biochim. Biophys. Acta 1108, 31–39.
[44] Chambers, T.C., McAvoy, E.M., Jacobs, J.W. and Eilon, G. (1990) J. Biol. Chem. 265, 7679–7686.
[45] Marsh, W. and Center, M.S. (1985) Biochem. Pharm. 34, 4180–4184.
[46] Epand, R.M. and Lester, D.S. (1990) Trends Pharm. Sci. 11, 317–320.
[47] Bates, S.E., Currier, S.J., Alvarez, M. and Fojo, A.T. (1992) Biochemistry 31, 6366–6372.
[48] Sikkema, J., De Bont, J.A.M. and Poolman, B. (1994) J. Biol. Chem. 269, 8022–8028.