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ORIGINAL ARTICLE

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P-glycoprotein inhibition by glibenclamide and related compounds

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Abstract Glibenclamide is well known to interact with the sulphonylurea receptor (SUR) and has been shown more recently to inhibit the cystic fibrosis transmembrane conductance regulator protein (CFTR), both proteins that are members of the ABC [adenosine 5′-triphosphate (ATP)-binding cassette] transporters. The effect of glibenclamide and two synthetic sulphonylcyanoguanidine derivatives (dubbed BM-208 and BM-223) was examined on P-glycoprotein, the major ABC transporter responsible for multidrug resistance (MDR) in cancer cells. To this end, we employed different cell lines that do or do not express P-glycoprotein, as confirmed by Western blotting: first, a tumour cell line (VBL600) selected from a human T-cell line (CEM) derived from an acute leukaemia; second, an epithelial cell line derived from a rat colonic adenocarcinoma (CC531^{mdr+}) and finally, a non tumour epithelial cell line derived from the proximal tubule of the opossum kidney (OK). Glibenclamide and the two related derivatives inhibited P-glycoprotein because firstly, they acutely increased [^{3}H]colchicine accumulation in P-glycoprotein-expressing cell lines only; secondly BM-223 reversed the MDR phenomenon, quite similarly to verapamil, by enhancing the cytotoxicity of colchicine, taxol and vinblastine and thirdly, BM-208 and BM-223 blocked the photoaffinity-labelling of P-glycoprotein by [^{3}H]azidopine. Furthermore, glibenclamide is itself a substrate for P-glycoprotein, since the cellular accumulation of [^{3}H]glibenclamide was low and substantially increased by addition of P-glycoprotein substrates (e.g., vinblastine and cyclosporine) only in the P-glycoprotein-expressing cell lines. We conclude that glibenclamide and two sulphonylcyanoguanidine derivatives inhibit P-glycoprotein and that sulphonylurea drugs would appear to be general inhibitors of ABC transporters, suggesting an interaction with some conserved motif.

Key words Multidrug resistance · Sulphonylurea · ABC transporters · Anti-cancer drugs

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Introduction

Tumour resistance to various cytotoxic drugs is a major cause of failure in cancer chemotherapy. This multidrug resistance (MDR) is mainly due to over-expression of the plasma membrane P-glycoprotein, the product of the MDR1 gene [4, 25]. This 170-kDa glycoprotein actively expels the hydrophobic anti-cancer agents from cells, thus maintaining their intracellular concentration at a low level and so inhibiting their anti-proliferative action [4, 25]. While over-expression of P-glycoprotein appears to result mainly from chemotherapy, its presence in several normal tissues or in some tumours at the time of the diagnosis in the absence of any treatment raises the question of the physiological or pathological substrate of P-glycoprotein. P-glycoprotein belongs to the superfamily of adenosine 5′-triphosphate (ATP)-binding cassette (ABC) transporters or traffic ATPases [13, 18], including also in humans the cystic fibrosis transmembrane conductance regulator protein (CFTR) and the sulphonylurea receptor (SUR). The CFTR is the chloride channel mutated in cystic fibrosis [30] and the SUR is linked in a macromolecular complex to an inward rectifier potassium channel, found in brain, heart, smooth muscle (SUR-2) [22] and pancreatic β–cells (SUR-1) where its closure is involved in insulin secretion [1, 21].

Glibenclamide binds to the SUR [1] and to the CFTR [34, 35] proteins and blocks their function. This drug may thus represent a general inhibitor for ABC transporters, in that it binds to some conserved motif. In the

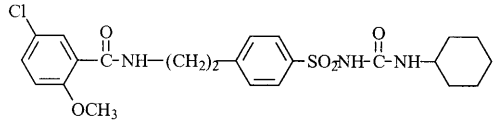
present study, the effects of glibenclamide and related derivatives were therefore tested on P-glycoprotein and significant inhibition was observed.

Materials and methods

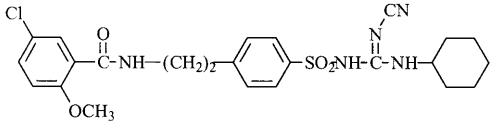
[3H]Colchicine (74 Ci/mmol) and [3H]glibenclamide (47 Ci/mmol) were purchased from New England Nuclear (Boston, Mass., USA); [3H]Azidopine (51 Ci/mmol) was obtained from Amersham (Buckinghamshire, England). Glibenclamide, colchicine, paclitaxel (taxol) and vinblastine were purchased from Sigma. Sulphonylcyanoguanidines derivatives of glibenclamide were synthesised according to general synthetic pathways previously described [27]. The chemical structure of the most potent derivatives is given in Fig. 1. Cyclosporine was generously provided by Sandoz Pharmaceuticals (Belgium). Leupeptin, trypsin inhibitor and pepstatin were purchased from Boerhinger Mannheim, Belgium and phenylmethylsulphonylfluoride (PMSF), iodoacetamide and (3-[4,5dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) (MTT) from Sigma (St. Louis, Miss., USA). Sodium dodecylsulphate (SDS) was from BioRrad, Belgium. All other chemicals were of the highest purity available, from Sigma or from Merck (Darmstadt, Germany).

The anti-P-glycoprotein monoclonal antibody C-219 was from Centocor (Malvern, Penn., USA), the anti-P-glycoprotein polyclonal antibody mdr (Ab-1) was purchased from EuroCalbiochem (Bierges, Belgium) and the anti-MRP-1 (MDR-associated protein) monoclonal antibody MRPm6 was obtained from Imtec (Antwerp, Belgium). Tissue culture media and reagents were purchased from Life Technologies (Merelbeke, Belgium) and Bio Whittaker (Verviers, Belgium).

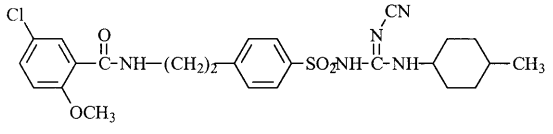
The following solutions were used: Buffer A: NaCl 130 mM, KCl 4 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, glucose 10 mM, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, tris(hydroxymethyl)aminomethane salt (TRIS-HEPES) 10 mM pH 7.4 and Buffer B: ethylenediaminetetraacetic acid (EDTA) 1 mM, TRIS-HEPES 10 mM pH 7.4. All drugs tested were dissolved in dimethylsulphoxide (DMSO), the final concentration of which was 0.1% in experiments with intact cells or 1% in experiments with plasma membrane fractions.



glibenclamide



BM-208



BM-223 (cis-trans)

Fig. 1 Chemical structures of glibenclamide derivatives. Glibenclamide: N^1-[4-[β-(2-methoxy-5-chlorobenzoylamino)ethyl]benzenesulphonyl]-N^2-cyclohexylurea; BM-208: N^1-[4-[β-(2-methoxy-5-chlorobenzoylamino)ethyl]benzenesulphonyl]-N^2-cyclohexylcyanoguanidine; BM-223: N^1-[4-[β-(2-methoxy-5-chlorobenzoylamino)ethyl]benzenesulphonyl]-N^2-[4-methylcyclohexyl]cyanoguanidine

Protein determination

Plasma membrane proteins were quantified by the bicinchoninic acid protein assay (BCA-Pierce Europe, The Netherlands) [40]. The protein content of each culture plate (35-mm diameter) was determined according to a modified procedure of the Lowry method [29].

Cell line culture

All cell lines were cultured at 37 °C in an atmosphere of 5% CO_2.

The non-tumour OK cell line, derived from the proximal tubule of the opossum kidney, was cultured as monolayer in Dulbecco's modified Eagle's Medium-F12 (DMEM-F12) containing 10% fetal calf serum (FCS) and penicillin-streptomycin (100 µg/ml-100 U/ml). The $CC531^{mdr+}$ cell line has been selected by progressive exposure to colchicine from its parental line, the CC531 [12, 38]. The latter line, which was derived from a colon carcinoma chemically induced in the rat, was shown to contain already substantial amounts of P-glycoprotein (data not shown) and therefore was not examined further. The $CC531^{mdr+}$ cell line was cultured as monolayer in the presence of 0.2 µM colchicine in DMEM supplemented with FCS 5%, $NaHCO_3$ 0.3%, L-asparagine 0.01 mM and L-glutamine 0.06 mM in the absence of antibiotics. For isotope uptake experiments, $CC531^{mdr+}$ and OK cells were seeded into six-well culture plate (35-mm diameter) at a density of 5×10^5 cells/well in 5 ml culture medium and grown to confluence in 3–4 days.

The CEM cell line, which is derived from a T-lymphocyte acute human leukaemia, was purchased from the American Tissue Culture Collection. The VBL600 line was selected from the parental CEM line by progressive exposure to vinblastine and maintained in the presence of 0.6 µg/ml vinblastine. This resulted in considerable expression of P-glycoprotein [3]. The CEM and VBL600 cell lines were cultured in suspension in RPMI 1640 with FCS 10%, L-glutamine 0.2 mM and penicillin-streptomycin (100 µg/ml-100 U/ml).

Growth inhibition assay (MTT cytotoxic assay)

VBL600 cells were washed with a vinblastine-free culture medium and seeded at a density of 2×10^4 viable cells/100 µl into 96-well microculture plates. The cells were incubated for 72 h with a cytotoxic agent alone (colchicine 0.2 µM, taxol 0.45 µM or vinblastine 1.1 µg/ml) or with 5 or10 µM of an agent inducing MDR reversal, either well-known (verapamil) or tested (glibenclamide, BM-208 and BM-223). Cell proliferation was evaluated by the MTT colourimetric method: the cells were incubated for 3 h in the presence of MTT (1 mg/ml) and the crystals formed were dissolved in isopropanol-HCl [28]. The microculture plates were shaken vigorously and the number of viable cells, which correlates to the difference of absorbances at 540 and 690 nm, was measured with a microplate-scanning spectrophotometer (Titertek Multiskan). At the concentration used, the cytotoxic agents did not affect the proliferation of VBL600 cells. For each drug tested, the inhibition of cell proliferation was expressed as a percentage of the absorbance measured in the presence and absence of the cytotoxic compound.

Uptake of [3H]colchicine and [3H]glibenclamide

Adherent cell lines:

Confluent monolayers of $CC531^{mdr+}$ and OK cells were cultured in 35-mm diameter culture plates and, for the $CC531^{mdr+}$ cells,

usually maintained in the presence of 0.2 µM colchicine, a colchicine-free medium was used for at least 12 h before starting the experiment. Each well was washed with 6 ml buffer A and preincubated for 10 min, at 37 °C, in 1 ml of the same buffer containing DMSO with or without a potential P-glycoprotein inhibitor. The cells were then incubated in 1 ml buffer A containing the isotope – either [^{3}H]colchicine (2.5 µCi/ml; with 35 nM colchicine) or [^{3}H]glibenclamide (2.5 µCi/ml; 52.5 nM glibenclamide) – and DMSO with or without the tested inhibitor. After 60 min, the radioactive medium was removed and the cells were washed rapidly with 5 ml ice-cold buffer A. The cellular radioactivity was extracted by acid treatment (1 ml HNO_3 0.1 N) and counted in a liquid scintillation medium (Ready Protein, Beckman).

Non adherent cell lines:

The CEM and VBL600 cells were cultured in suspension in 175-cm^2 flasks at concentrations of 0.5–1×10^6 cells/ml. The culture medium was removed after centrifugation and the cells washed with 30 ml buffer A and suspended in 1.5 ml of the same buffer. For each assay, 1.5×10^6 viable cells were preincubated for 10 min at 37 °C, in 300 µl buffer A in the presence of DMSO with or without a tested inhibitor. An aliquot (300 µl) of the same buffer containing the isotope – either [^{3}H]colchicine (final concentration 1.25 µCi/ml; 17.5 nM colchicine) or [^{3}H]glibenclamide (final concentration 0.4 µCi/ml, 10 µM glibenclamide) – and DMSO with or without inhibitor were added to the cell suspension. After 60 min incubation at 37 °C, the cells were centrifuged at 8,700 g for 3 min at 4°C (Microfuge B, Beckman, Palo Alto, Calif., USA) and the radioactive medium removed. The cell pellet was washed with 1.5 ml ice-cold buffer A, suspended in 0.5 ml of the same buffer and radioactivity counted in a liquid scintillation medium.

Preparation of plasma membrane fractions from cell lines

Plasma membrane fractions from different cell lines were prepared by differential centrifugation. The OK and CC531^{mdr+} cells were cultured at confluence in 175-cm^2 flasks. The cells were rinsed with ice-cold phosphate buffer and incubated for 30 min at 4°C with 20 ml hypotonic buffer B containing desoxyribonuclease I (DNaseI) 5 µg/ml and protease inhibitors (PMSF 0.1 mM, leupeptin 5 µg/ml, pepstatin 0.2 µg/ml, trypsin inhibitor 10 µg/ml and iodoacetamide 0.1 mM). The cells were scrapped and homogenised in a dounce tissue grinder (Wheaton-Millville, New Jersey, USA).

The CEM and VBL600 cells were cultured in flasks of 175 cm^2 at a concentration of about 1×10^6 cells/ml. The culture medium was removed after centrifugation and the pellet was suspended in an ice-cold hypotonic buffer containing $NaHCO_3$ 1 mM and protease inhibitors. The cells were homogenised in a dounce tissue grinder.

Homogenates from all cell lines treated according to the same procedure. After low-speed centrifugation (1,000 g, 15 min, 4 °C) to separate nuclei and cellular debris, the supernatant was centrifuged at high speed (48,000 g, 30 min, 4 °C). The resulting pellet was suspended in buffer B and centrifuged again at high speed (48,000 g, 30 min, 4°C). The "crude plasma membrane fraction" obtained was suspended in the same buffer and aliquots were stored at –80 °C until use.

Immunodetection of P-glycoprotein and MRP-1

Plasma membrane proteins were solubilised in 1 volume of a TRIS-HCl electrophoresis buffer 125 mM pH 6.8, containing glycerol 20%, SDS 4% and bromphenol blue 0.005%. The proteins (5–50 µg) were loaded onto a 7.5% polyacrylamide gel (PAGE) containing SDS (SDS-PAGE) and transferred onto a nitrocellulose membrane (Hybond ECL, Amersham) by electroblotting for 1 h at 20 V in TRIS 12 mM, glycine 96 mM, pH 8.3. The resulting blots were blocked (16 h at 4 °C) with a TRIS-HCl buffer 10 mM pH 8.0, containing NaCl 150 mM, Tween 20 0.05% and non-fat dry milk 8% (TBST blocking buffer) and incubated for 3 h at 25 °C with an anti-P-glycoprotein antibody in blocking buffer, either C-219 (0.5 µg/ml) or mdr (Ab-1) (0.4 µg/ml) or with the anti MRP-1 antibody (0.2 µg/ml) in blocking buffer.

Specific immunocomplexes were detected using a second biotinylated sheep anti-mouse or donkey anti-rabbit antibody (Amersham; 1:4,000 dilution) and a streptavidin-biotinylated horseradish peroxidase complex (Amersham; 1:4,000 dilution). After extensive washing with TBST, membranes were incubated with the enhanced chemiluminescent reagent (ECL, Amersham) and autoradiographed for 20–60 s. The specificity of the reaction with mdr (Ab-1) was assessed by competition experiments using the mdr peptide prepared for raising this antibody. This peptide (40 µg/200 µl PBS) was incubated for 16 h at 4 °C with 4 µg mdr (Ab-1) antibody prior to its dilution in blocking buffer. The molecular weights were estimated using the BenchMark Protein Ladder (Life Technologies, Belgium).

Photoaffinity labelling of plasma membranes P-glycoprotein by [^{3}H]azidopine

Plasma membrane proteins (75 µg) were preincubated in a Packard vial for 15 min at room temperature in 200 µl buffer B containing DNaseI 5 µg/ml, protease inhibitors and a tested inhibitor (1:100 vol:vol). [^{3}H]Azidopine (2 µCi, 0.16 µM) was added in 50 µl of the same buffer and the membranes incubated in the dark for 45 min at room temperature. The membranes were then irradiated in the dark at 254 nm (UV lamp 15 W, Vilber Lourmat) at 4 °C for 20 min at a distance of 20 cm. Following transfer to an Eppendorf tube, the membranes were the centrifuged (15,800 *g*, 30 min, 4 °C, Microfuge E, Beckman, Palo Alto, Calif., USA) and washed with 1 ml ice-cold buffer B. This procedure was repeated twice and the final membrane pellet was suspended in 15 µl of the same ice-cold buffer and stored at –80 °C until use.

Labelled plasma membrane proteins (70 µg) were fractionated by SDS-PAGE as described above. The proteins were fixed with 10% acetic acid, 40% methanol in water for 1 h at 25 °C followed by incubation with 1 M salicylic acid, 40% ethanol in water for 1 h at 25 °C. After washing with water, the gel was dried and autoradiographed for 20 days at –80 °C in a Kodak X-omatic cassette.

Presentation of results

Experiments were performed at least in triplicate. In each figure, the values are presented as means±SE. SE are not shown when the values are smaller than the symbol used.

Results

Accumulation of P-glycoprotein substrates by the different cell lines

[^{3}H]Colchicine accumulation

Accumulation of [^{3}H]colchicine by the different cell lines was measured in the absence or presence of well-known competing substrates for the P-glycoprotein, vinblastine 100 µM, cyclosporine 50 µM and verapamil 100 µM (Fig. 2). These high concentrations were chosen so as to achieve nearly 100% P-glycoprotein inhibition. The incubation medium contained either 35 nM colchicine for the CC531^{mdr+} and OK cell lines or 17.5 nM colchicine for the VBL600 and CEM cell lines. The cellular accumulation of [^{3}H]colchicine, at 60 min, was ex-

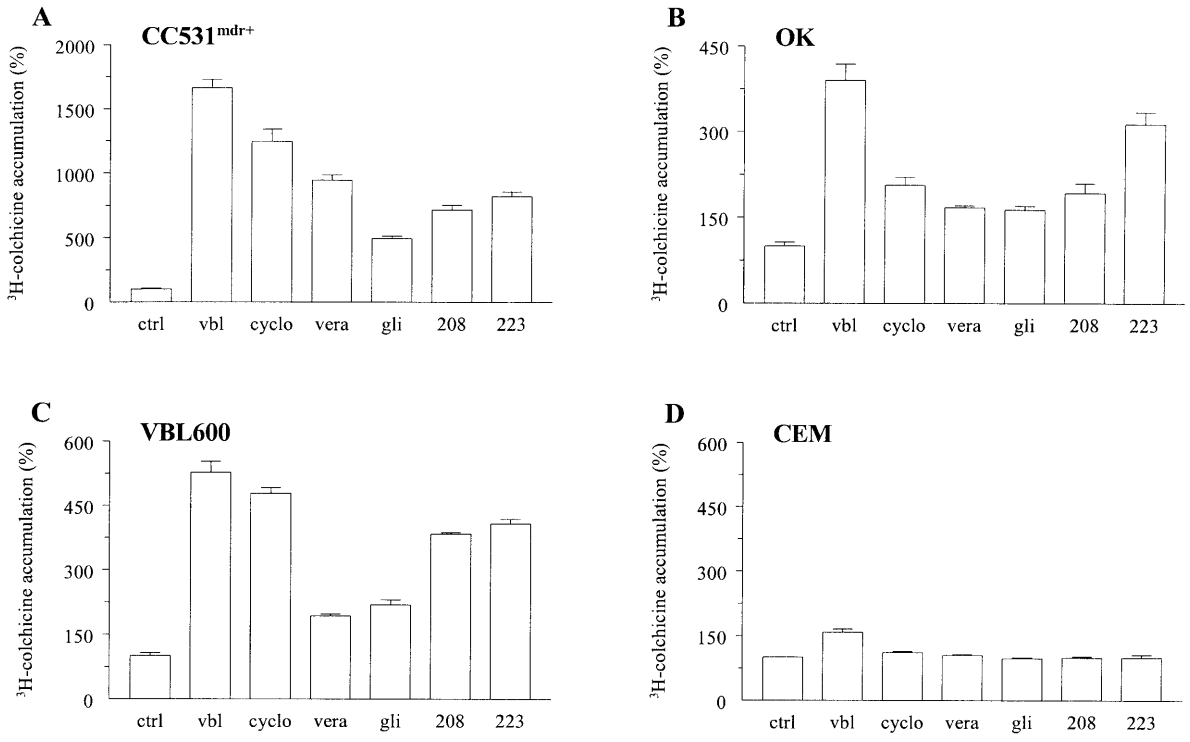


Fig. 2A–D Effect of inhibitors on [3H]colchicine accumulation in the different cell lines. [3H]Colchicine uptake was measured after 60 min in CC531^{mdr+} (**A**), OK (**B**), VBL600 (**C**) and CEM (**D**) cell lines, in the absence or presence of one of the following potential inhibitors: vinblastine (*vbl*) 100 µM, cyclosporine (*cyclo*) 50 µM, verapamil (*vera*) 100 µM, glibenclamide (*gli*) 100 µM, BM-208 (*208*) 100 µM, BM-223 (*223*) 100 µM. [3H]colchicine accumulation is expressed as a percentage of its incorporation under control conditions (*ctrl*), i.e. in the absence of inhibitor

Table 1 Effect of antiseptic sulphonamides (100 µM) on [3H]colchicine accumulation in CC531^{mdr+} and OK cell lines. Results are expressed as a percentage of that in control medium at 60 min (*n*=3)

Sulphonamide drugs	Colchicine accumulation	
	CC531^{mdr+} cells	OK cells
Sulphamethoxazole	97.7±7.4	97.6±3.2
Sulphadiazine	112.1±11.3	102.3±3.1

pressed as the percentage of the accumulation measured in the absence of inhibitor, i.e. under control conditions. As expected, inhibitors of P-glycoprotein considerably increased [3H]colchicine accumulation in CC531^{mdr+}, OK and VBL600 cells, but not in CEM cells (which do not express significant amounts of P-glycoprotein), as confirmed here: see Fig. 5A and B.

Glibenclamide and the related derivatives BM-208 and BM-223 (Fig. 1), tested under the same conditions increased [3H]colchicine accumulation similarly in all cells except CEM (Fig. 2). In contrast, currently used antiseptic sulphonamides such as sulphamethoxazole and sulphadiazine failed to modify [3H]colchicine accumulation (Table 1).

Although the level of [3H]colchicine accumulation varied among the different cell lines, the pattern of relative effectiveness of the P-glycoprotein inhibitors was quite similar (Fig. 2). In the absence of inhibitor, the CC531^{mdr+} and VBL600 cells incorporated the lowest

Table 2 Cellular accumulation of [3H]colchicine and of [3H]glibenclamide at 60 min, corresponding to the control values, i.e. 100% in Figs. 2 and 3. The concentration of colchicine and glibenclamide in the incubation medium is given for each condition

Cell lines	Colchicine		Glibenclamide	
	Concentration (nM)	Cellular uptake (pmol/mg)	Concentration (nM)	Cellular uptake (pmol/mg)
Adherent cell lines				
CC531^{mdr+}	35	0.11±0.01 (17)	52.5	0.11±0.02 (6)
OK	35	0.55±0.04 (6)	52.5	2.61±0.25 (3)
Non-adherent cell lines				
VBL600	17.5	0.11±0.01 (3)	10,000	920±10 (3)
CEM	17.5	0.77±0.01 (3)	10,000	1560±10 (3)

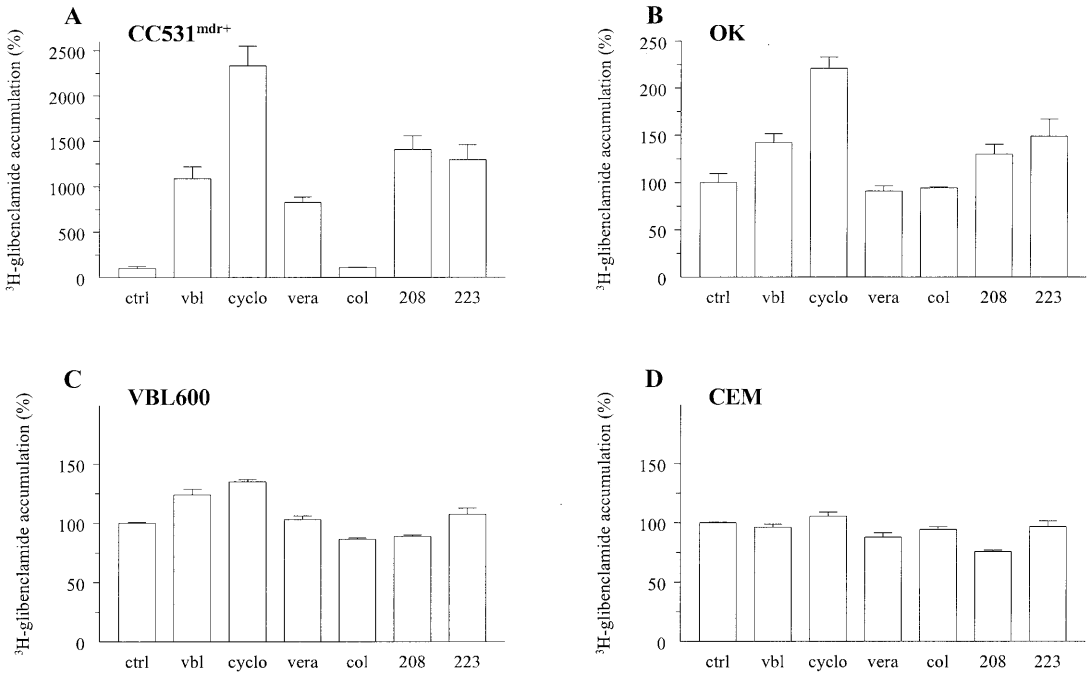


Fig. 3A–D Effect of inhibitors on [^{3}H]glibenclamide accumulation in the different cell lines. [^{3}H]Glibenclamide uptake was measured after 60 min, at 52.5 nM glibenclamide, in CC531^{mdr+} (**A**) and OK (**B**) cells, in the absence or presence of the following inhibitors: vbl 100 µM, cyclo 50 µM, vera 100 µM, colchicine (*col*) 100 µM, 208 100 µM and 223 100 µM. Similar experiments were performed on VBL600 (**C**) and CEM (**D**) cells at 10 µM glibenclamide to circumvent high, non-specific binding. [^{3}H]glibenclamide accumulation is expressed as a percentage of its incorporation under control conditions (*ctrl*), i.e. in the absence of inhibitor

amount of [^{3}H]colchicine (see Table 2), suggesting that those cell lines contain higher amounts of functional P-glycoprotein.

[^{3}H]Glibenclamide accumulation

The accumulation of [^{3}H]glibenclamide was also measured in the same cell lines in the presence of the inhibitors tested above and colchicine, 100 µM (Fig. 3). The incubation medium contained either 52.5 nM glibenclamide for the CC531^{mdr+} and OK cells or 10 µM glibenclamide for the VBL600 and CEM cells. In the experimental conditions used for the non adherent cell lines, a high non-specific binding of [^{3}H]glibenclamide was observed at lower concentrations, as reported for some hydrophobic P-glycoprotein inhibitors [24]. The level of [^{3}H]glibenclamide accumulation varied among the cell lines but the patterns of relative potency of the different inhibitors were nearly identical. Since the experiments with the VBL600 and CEM cells were performed in the presence of 10 µM glibenclamide, the concentration ratio of each inhibitor to glibenclamide was less favourable and the inhibitory potency was significantly reduced. As shown in Table 2, the accumulation of [^{3}H]glibenclamide under the control conditions clearly indicated that the CC531^{mdr+} cells contained more functional P-glycoprotein than the OK cells and that the VBL600 contained more functional P-glycoprotein than the CEM cells.

Growth inhibition assay: reversal of P-glycoprotein MDR by inhibitors

The cytotoxic assay allowed evaluation of the chronic inhibition of P-glycoprotein (after 72 h) by glibenclamide and related compounds. VBL600 cells were cultured in the presence of different cytotoxic agents: colchicine (0.2 µM), taxol (0.45 µM) or vinblastine (1.1 µg/ml), i.e. at concentrations that do not significantly affect the proliferation of these cells. The cytotoxic effect of these agents was similarly restored by the addition of verapamil or BM-223, indicating the reversal of the MDR phenomenon (Fig. 4A–C). In contrast, glibenclamide and BM-208 did not affect cell proliferation substantially, indicating that in chronic exposure, the latter agents were not able to reverse the MDR phenomenon.

Expression of P-glycoprotein and MRP-1 in the cell lines: Western blot analysis

The presence of P-glycoprotein in plasma membrane fractions prepared from the different cell lines was demonstrated by Western blot analysis using antibodies raised against epitopes of the human P-glycoprotein (Fig. 5A, B). When the monoclonal antibody C-219 was used, a strong 170-kDa band was detected in the VBL600 cell line (5 µg protein). This band was completely absent in CEM cells, even though a tenfold higher amount of protein was loaded (50 µg). The 170-kDa band was present, although weaker, in the CC531^{mdr+} and OK cells (25 µg

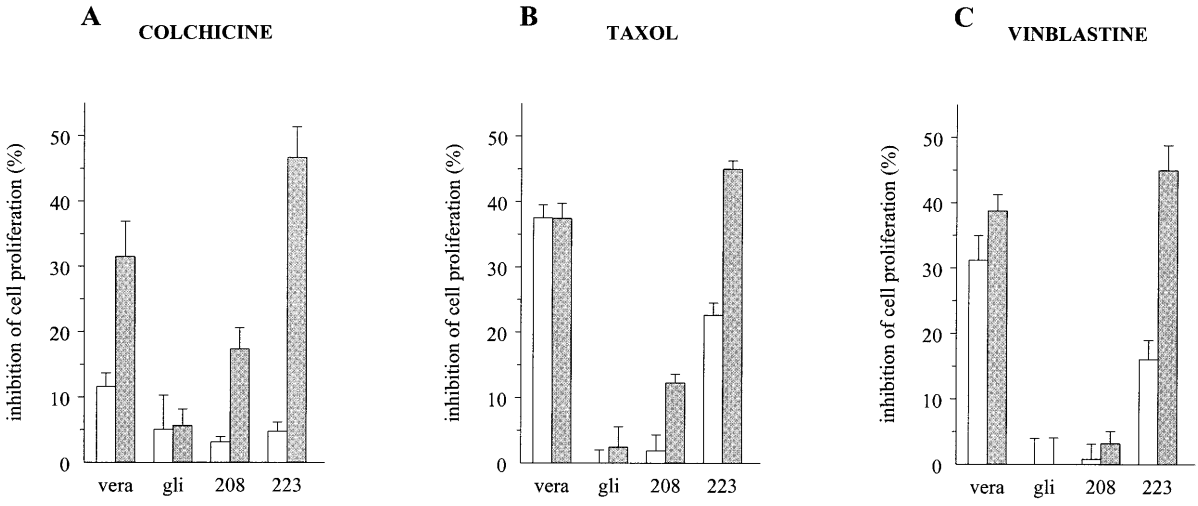


Fig. 4A–C Chemosensitization of VBL600 cells by different inhibitors. The VBL600 cells were cultured for 72 h in the presence of colchicine 0.2 µM (**A**), taxol 0.45 µM (**B**) and vinblastine 1.1 µg/ml (**C**) in the presence of vera, gli, 208 and 223 at 5 (*open bars*) and 10 µM (*closed bars*). The inhibition of cell proliferation was measured as described in the Methods (*n*=3–9)

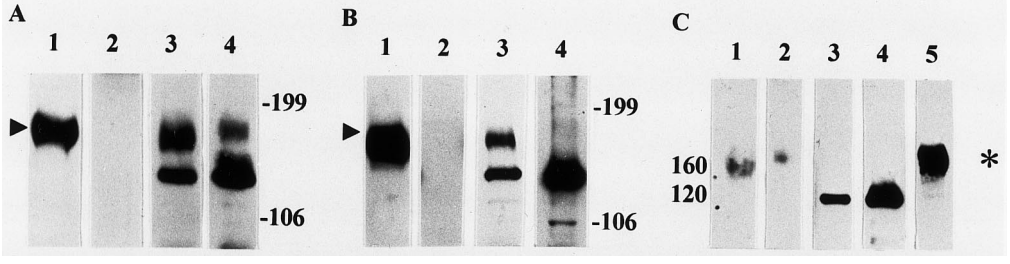


Fig. 5A–C P-glycoprotein and multidrug resistance-associated protein-1 (MRP-1) immunodetection in cell membranes. Membrane protein (5–50 µg) were separated by reducing sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% acrylamide gels), transferred and detected as described in the Methods using anti-P-glycoprotein antibodies: C-219 monoclonal antibody at 0.5 µg/ml (**A**) or anti-mdr (Ab-1) polyclonal antibody at 0.4 µg/ml (**B**), or using the anti-MRP-1 monoclonal antibody (MRPm6) at 0.2 µg/ml (**C**). *Arrows* indicate the P-glycoprotein at 170 kDa, * indicates the MRP-1 at 190-kDa. *Lane 1*: VBL600 membranes: 5 (**A**), 28 (**B**) and 25 (**C**) µg. *Lane 2*: CEM membranes: 50 (**A**), 30 (**B**) and 25 (**C**) µg. *Lane 3*: CC531^{mdr+} membranes: 25 (**A**), 31 (**B**) and 25 (**C**) µg. *Lane 4*: OK membranes: 25 (**A**), 30 (**B**) and 25 (**C**) µg. *Lane 5*: PSN1/ADR membranes: 5 µg (**C**). The PSN1/ADR cell line is derived from a human pancreatic adenocarcinoma. It was selected by progressive exposure to doxorubicin, which induces over-expression of the MRP-1 protein

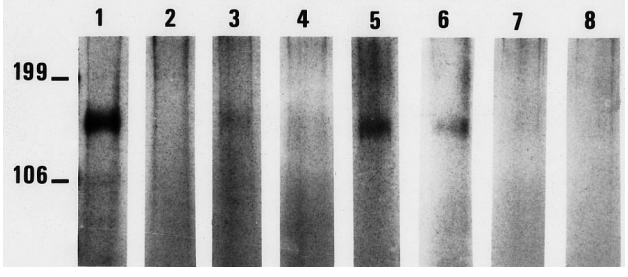


Fig. 6 Effect of inhibitors on [^{3}H]azidopine photolabelling of P-glycoprotein. Plasma membrane proteins (70 µg) from VBL600 (*lanes 1, 3–8*) or CEM (*lane 2*) cells were UV-labelled with [^{3}H]azidopine as described in the Methods, fractionated by SDS-gel electrophoresis and detected by autoradiography. Samples contained buffer only (*lanes 1–2*), vinblastine 100 µM (*lane 3*), cyclosporine 50 µM (*lane 4*), verapamil 100 µM (*lane 5*), glibenclamide 300 µM (*lane 6*), BM-208 100 µM (*lane 7*) and BM-223 100 µM (*lane 8*)

protein in each lane). With the polyclonal mdr (Ab-1) antibody, a similar but weaker pattern was observed in all cells except the OK cell line, in which the 170-kDa band was lacking. The bands detected, at around 130-kDa, were non-specific as they remained unchanged when the first antibody was omitted (data not shown) or when it was replaced by an anti-MRP-1 antibody, as observed in Fig. 5C (lanes 3 and 4). The over-expression of P-glycoprotein in the VBL600 or in the CC531^{mdr+} cell line was not accompanied by over-expression of MRP-1 protein (Fig. 5C). A weak band was detected at around 190-kDa with the anti-MRP-1 antibody, only in the VBL600 and CEM cells, while a strong 190-kDa band was observed in PSN1/ADR cells, well-known to over-express MRP-1 [39].

Photoaffinity labelling of P-glycoprotein

Membrane fractions of VBL600 cells were incubated with [^{3}H]azidopine with or without a 320-fold molar excess of cyclosporine (50 µM), a 640-fold molar excess of vinblastine, verapamil, BM-208 and BM-223 (100 µM) or a 1920-fold molar excess of glibenclamide (300 µM). [^{3}H]Azidopine labelled a 170-kDa band in VBL600 but not in CEM membranes; the latter do not express P-glycoprotein (Fig. 6). The [^{3}H]azidopine labelling was completely inhibited by cyclosporine, BM-208 and BM-223, but only partially by verapamil and vinblastine. In the case of glibenclamide however, the large excess of drug required indicated a weak or a non-specific effect. These results suggest competition between azidopine and most of the drug tested for binding to P-glycoprotein.

Discussion

The failure of cancer chemotherapy is a major clinical problem. It is linked to the development of resistance to the drug used but also to cross-resistance to different, chemically unrelated cytotoxic agents. This phenomenon has been called MDR [13]. It is usually explained by the over-expression of P-glycoprotein, a membrane ATPase which actively extrudes cytotoxic agents from the cell, thereby preventing their lethal action. It should be recognised that MDR in some forms of cancer may have other causes: over-expression of other proteins related to P-glycoprotein, like MRP [2] or the lung resistance-related Protein (LRP) [33], variations in activities of topoisomerases [13] or even over-expression of some oncogene [41].

Two isoforms of P-glycoprotein, called mdr1 and mdr2, are normally expressed in humans with a tissue-specific distribution [13]. These isoforms are encoded by two different genes but only mdr1 is over-expressed in tumour cells. This 170-kDa protein comprises two transmembrane domains (each containing six transmembrane helices) and two cytoplasmic nucleotide binding domains that exhibit ATP binding motifs with consensus sequences common to all members of the ABC family [25]. In humans, three members of this superfamily – P-glycoprotein, SUR [1] and CFTR [30] – have recently received considerable attention as they are believed to control other membrane proteins. SUR and CFTR are inhibited by glibenclamide and related sulphonylurea derivatives, although with different affinities [22, 34, 35]. Glibenclamide has the highest affinity (in the nanomolar range) for the pancreatic β–cell SUR 1 and is currently used as a potent hypoglycaemic agent, usually devoid of significant side effects. At therapeutic concentrations glibenclamide thus only interferes with one isoform of SUR. The present work was designed to test whether glibenclamide and synthetic sulphonylcyanoguanidine derivatives inhibit P-glycoprotein and can be considered as general ABC transporter inhibitors.

To this end, several cell lines were used: the well-characterised human VBL600 cell line over-expressing P-glycoprotein and selected from its parental CEM cell line by progressive exposure to vinblastine, [3, 9, 11]; the CEM cell line in which P-glycoprotein expression was undetectable; the CC531^{mdr+} line that over-expresses P-glycoprotein and is derived from a rat colon carcinoma by colchicine selection [8, 12] and the non-tumour OK cell line expressing P-glycoprotein constitutively as does the proximal tubule of the human kidney [19, 26, 37].

The expression of P-glycoprotein was confirmed by Western blot analysis as a 170-kDa band. Two different antibodies were employed: the monoclonal C-219 and the polyclonal anti mdr (Ab-1). The latter failed to detect the P-glycoprotein band in OK cells, this is probably related to species differences. P-glycoprotein was never detected in the CEM cells, suggesting that this cell line barely express mdr1, as confirmed here using functional tests. The process of MDR selection in the VBL600 and CC531^{mdr+} cells was not associated with over-expression of the MRP-1 protein. No 190-kDa band was observed in the CC531^{mdr+} and the OK cells and only a faint band was observed at around 190-kDa in the VBL600 and the CEM cells. Thus, in these two cell lines, the expression of MRP-1 is low and not very different, contrasting with the sharp difference observed in P-glycoprotein expression (Fig. 5A, B, lanes 1 and 2).

The potential inhibition of P-glycoprotein by glibenclamide and sulphonylurea derivatives was studied in the different cell lines. To this end, the steady state cellular accumulation of [^{3}H]colchicine at 60 min was measured in the presence or absence of well-known P-glycoprotein substrates such as vinblastine [13, 25], cyclosporine [10, 13] and verapamil [25, 31]. The percentage increase in colchicine uptake was the highest in CC531^{mdr+} cells although obvious for all cell lines excepting CEM cells. The pattern of relative potency of the different inhibitors tested was similar for all cell lines and, of interest, the potency of the sulphonylcyanoguanidine derivatives (BM-208 and BM-223) in increasing colchicine accumulation was similar or higher than that of verapamil.

The question was then addressed whether glibenclamide itself is actually a substrate for P-glycoprotein. In the CC531^{mdr+} and OK cell lines, the steady state accumulation of [^{3}H]glibenclamide was measured and shown to be quite significantly increased by P-glycoprotein substrates including BM-208 and BM-223. For the study of cells in suspension (VBL600 and CEM cells) the concentration of cold glibenclamide was increased to 10 µM (instead of 52.5 nM for cells grown in monolayers) to circumvent the high, non-specific background associated with the technique. No difference in [^{3}H]glibenclamide accumulation was observed in CEM cells while there was a significant increase with vinblastine and cyclosporine in VBL600 cells. This supports the conclusion reached with the adherent cell lines, that glibenclamide is itself a substrate of P-glycoprotein.

[^{3}H]glibenclamide accumulation in the different cell lines tested was not increased by colchicine, while [^{3}H]colchicine accumulation was well inhibited by glibenclamide, suggesting a higher affinity of P-glycoprotein

for glibenclamide than for colchicine. Indeed, the affinity of P-glycoprotein for the various drugs transported is related to some defined amino acid(s) in or near the transmembrane segments, as several specific mutations within these regions induce elective modification in the transport rate [6, 15].

In the past, [^{3}H]azidopine has been used to UV-photolabel P-glycoprotein. Such photolabelling can be impaired by competing substrates [5, 14, 31]. In the present study, prominent 170-kDa band was observed when VBL600, but not CEM, membranes were photolabelled with [^{3}H]azidopine. This band completely disappeared on simultaneous incubation with either cyclosporine, BM-208 and BM-223 but only partially with verapamil and vinblastine. The excess of substrate used here (300- to 600-fold) to compete with [^{3}H]azidopine UV-labelling is in accordance with the range of concentrations previously reported (200- to 1,000-fold) [7, 20, 32]. Photolabelling of P-glycoprotein was inhibited only by a large excess of glibenclamide (about 2,000-fold) and even then not completely. This weak or non-specific effect does however not rule out the possibility that glibenclamide is a P-glycoprotein substrate (see above) as several other P-glycoprotein substrates (colchicine, adriamycin and actinomycin D) also fail to interfere with [^{3}H]azidopine labelling [32].

The reversal of MDR was demonstrated in the VBL600 cell line cultured for 72 h in the presence of different cytotoxic agents (colchicine, taxol or vinblastine). Cell proliferation was estimated using a growth inhibitory assay [16, 17, 23, 36]. The sulphonylcyanoguanidine derivative BM-223 behaved as a chemosensitizer equipotent to verapamil, restoring the cytotoxicity of colchicine, taxol and vinblastine. This observation could be of potential clinical importance to reverse MDR if derivatives were to exhibit a higher affinity for P-glycoprotein than for both SUR and CFTR.

In summary the present study demonstrates that glibenclamide and some structurally related sulphonylcyanoguanidines inhibit P-glycoprotein and appear to be general inhibitor of ABC transporters. It will be interesting in the future to map the glibenclamide binding site(s) in different ABC transporters to establish possible correspondence with some conserved motif(s).

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