The acridonecarboxamide GF120918 potently reverses P-glycoprotein-mediated resistance in human sarcoma MES-Dx5 cells

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Summary

The doxorubicin-selected, P-glycoprotein (P-gp)-expressing human sarcoma cell line MES-Dx5 showed the following levels of resistance relative to the non-P-gp-expressing parental MES-SA cells in a 72 h exposure to cytotoxic drugs: etoposide twofold, doxorubicin ninetofold, vinblastine tenfold, taxotere 19-fold and taxol 94-fold. GF120918 potently reversed resistance completely for all drugs. The \( K_{i} \) of GF120918 to reverse resistance of MES-Dx5 cells were: etoposide 7 ± 2 nM, vinblastine 19 ± 3 nM, doxorubicin 21 ± 6 nM, taxotere 57 ± 14 nM and taxol 91 ± 23 nM. MES-Dx5 cells exhibited an accumulation deficit relative to the parental MES-SA cells of 35% for \([^{3}H]\)-vinblastine, 20% for \([^{3}H]\)-taxol and \([^{14}C]\)-doxorubicin. The \( EC_{50} \) of GF120918, to reverse the accumulation deficit in MES-Dx5 cells, ranged from 37 to 64 nM for all three radiolabelled cytotoxics. \([^{3}H]\)-vinblastine bound saturably to membranes from MES-Dx5 cells with a \( K_{d} \) of 7.8 ± 1.4 nM and a \( B_{\text{max}} \) of 5.2 ± 1.6 pmol mg\(^{-1}\) protein. Binding of \([^{3}H]\)-vinblastine to P-gp in MES-Dx5 membranes was inhibited by GF120918 (\( K_{i} = 400 ± 140 \text{ nM} \)). The novel acridonecarboxamide derivative GF120918 potently overcomes P-gp-mediated multidrug resistance in the human sarcoma cell line MES-Dx5. Detailed analysis revealed that five times higher GF120918 concentrations were needed to reverse drug resistance to taxol in the cytotoxicity assay compared to doxorubicin, vinblastine and etoposide. An explanation for this phenomenon had not been found.

Keywords: multidrug resistance; P-glycoprotein inhibitor; GF120918; MES-Dx5 cells

Materials and Methods

Materials

McCoy’s 5A cell culture medium was purchased from Sigma (Poole, UK) and all other cell culture reagents from Gibco-BRL.
Figure 1 Structure of GF120918

(Paisley, UK). MES-SA and MES-Dx5 were obtained from the American Type Tissue Culture Collection (Rockville, MD, USA) and MCF-7/ADR cells from the European Collection of Cell Culture (Salisbury, UK). [³H]-vinblastine (11.4–13.5 Ci mmol⁻¹) and [¹⁴C]-doxorubicin (60 mCi mmol⁻¹) were purchased from Amersham (Little Chalfont, UK). [³H]-taxol (19.3 Ci mmol⁻¹) was obtained from the National Cancer Institute (Bethesda, MD, USA). [³H]-taxol and [¹⁴C]-doxorubicin were aliquoted and not frozen after thawing. Doxorubicin, etoposide, taxol, vinblastine and cyclosporin A were supplied by Sigma (Poole, UK). Taxotere was a gift from Rhône-Poulenc Rorer (Paris, France). Verapamil and GF120918 (Figure 1) were supplied by GlaxoWellcome (USA). CP100356 was a gift from Dr Kajiji, Pfizer (Groton, CT, USA). GF120918 was a gift from Rhône-Poulenc Rorer (Paris, France). Doxorubicin, etoposide, taxol, vinblastine and cyclosporin A were supplied by Sigma (Poole, UK). Taxotere was a gift from Rhône-Poulenc Rorer (Paris, France). Verapamil and GF120918 (Figure 1) were supplied by GlaxoWellcome (USA). CP100356 was a gift from Dr Kajiji, Pfizer (Groton, CT, USA).

Cytotoxicity assay

The tetrazolium dye based MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983) was used to assess cytotoxicity. MES-SA or MES-Dx5 cells were seeded into flat-bottom 96-well plates at 2000 cells per well and grown for 24 h in drug-free medium. Cells were incubated for 72 h with cytotoxic agents ± GF120918. A total of 20 μl of MTT dye (5 mg ml⁻¹ phosphate-buffered saline (PBS)) was added and incubated for 4 h at 37°C. All medium was removed and the formazan crystals dissolved in 100 μl DMSO. The absorption was measured in a spectrophotometer at a wavelength of 550 nm.

Radiolabelled drug accumulation assay

Drug accumulation assays were performed as previously described (Ferry et al, 1995). Briefly, 80 × 10⁴ MES-SA or MES-Dx5 cells ml⁻¹ were plated into each well of a 12-well plate and incubated for 24 h. McCoy’s 5A medium was replaced with 1 ml uptake medium per well (McCoy’s 5A medium without supplements and added 5 mM glucose and 5 mM magnesium chloride (MgCl₂)). The cells were incubated in a shaking waterbath at 37°C in near darkness. GF120918 (1 μM) in 10 μl of DMSO or 10 μl DMSO for the control were added to each well. [³H]-vinblastine (= 1 nM), [³H]-taxol (= 0.5 nM) or [¹⁴C]-doxorubicin (= 20 nM) were added at a volume of 10 μl per well which commenced the assay and incubated for 30 min to 4 h at 37°C. The reaction was stopped by removal of the uptake medium and addition of 1 ml liquid scintillant. Radioactivity was quantitated by liquid scintillation counting.

Preparation of cell surface membranes

MES-SA and MES-Dx5 cell surface membranes were prepared by a method as previously described (Ferry et al, 1992). Ten 80 cm² flasks of cells were grown to confluence (equivalent to 5 × 10⁶ cells). After removal of medium and addition of ice-cold buffer A (50 mM Tris-HCl, pH 7.4, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.1 mM EDTA) the cells were harvested with a rubber policeman. The cell suspension was centrifuged (3500 g, 10 min (3000 g, 4°C for 3 min) and the supernatant discarded. The packed cell volume was measured and 1 ml pellet was resuspended in 9 ml ice-cold buffer A. Cells were homogenized with a polytron (6 × 30 s, setting 6). The crude homogenate was centrifuged for 10 min (3500 g, 4°C) in a Sorvall centrifuge and the supernatant discarded. The packed cell volume was measured and 1 ml pellet was resuspended in 9 ml ice-cold buffer A. Cells were homogenized with a polytron (6 × 30 s, setting 6). The crude homogenate was centrifuged for 10 min (3500 g, 4°C) in a Sorvall centrifuge and the supernatant discarded. The packed cell volume was measured and 1 ml pellet was resuspended in 9 ml ice-cold buffer A. Cells were homogenized with a polytron (6 × 30 s, setting 6). The crude homogenate was centrifuged for 10 min (3500 g, 4°C)). Doxorubicin, etoposide, taxol, vinblastine and cyclosporin A were supplied by Sigma (Poole, UK). Taxotere was a gift from Rhône-Poulenc Rorer (Paris, France). Verapamil and GF120918 (Figure 1) were supplied by GlaxoWellcome (USA). CP100356 was a gift from Dr Kajiji, Pfizer (Groton, CT, USA).

Gel electrophoresis and Western blotting

A 6% sodium dodecyl sulphate (SDS) gel (Sambrook et al, 1989) was loaded with 10–100 μg membrane protein after denaturation with gel sample buffer and run at 12 mA overnight. The protein was transferred over 4 h onto a nitro-cellulose blotting membrane. Non-specific binding was blocked with 5% milk powder in PBS and Tween. Incubation with the primary monoclonal antibody NCL-JSB-1 (1:500 in 5% milk in PBS/Tween) lasted 16 h at 4°C. The secondary antibody, mouse peroxidase IgG (1:1000 in 5% milk in PBS/Tween), was added for 1 h and the protein was visualized using the ECL protein detection reaction.
Membrane binding assay

Binding of [\(^{3}H\)]-vinblastine to membrane preparations was performed as previously described (Ferry et al, 1992). Briefly, 50 \(\mu\)l buffer B (50 mM Tris-HCl, pH 7.4 and 0.1 mM PMSF), 50 \(\mu\)l drug diluted in buffer B, 50 \(\mu\)l [\(^{3}H\)]-vinblastine (5–7 nm) were incubated with 100 \(\mu\)l membrane preparation (protein concentration 50–60 \(\mu\)g per assay) for 3 h in near darkness at 21°C. The total assay volume was 0.25 ml. The assay was stopped by adding 3.5 ml ice-cold washbuffer (20 mM Tris–HCl, pH 7.4 and 20 mM MgCl\(_{2}\)) followed by rapid filtration through a Whatman GF/C filter (pre-wetted with 50 mM Tris–HCl and 0.1% albumin) to separate bound from free radioactivity. The filter was washed twice with 5 ml washbuffer to remove free radioactivity, dried and added to scintillation vials filled with 3 ml scintillant. Retained, bound radioactivity was measured with a liquid scintillation counter.

Rhodamine dye accumulation and efflux assay measured by FACS analysis

The rhodamine (Rh123) dye accumulation assay was performed as previously described (Davies et al, 1996). Briefly, 1 \(\times\) 10\(^{6}\) MES-SA or MES-Dx5 cells were seeded into 55-mm Petri dishes and allowed to become adherent overnight at 37°C. The medium was replaced with 5 ml additive-free medium and incubated with 100 \(\mu\)l Rh123 for 20 min at 37°C in darkness. The cells were washed with PBS (37°C). Control cells were harvested before the cell pellet was washed in ice-cold PBS and resuspended in 1 ml ice-cold PBS and propidium iodate (5 \(\mu\)g ml\(^{-1}\)), incubated for 10 min on ice followed by a wash in ice-cold PBS and centrifugation (3000 \(g\), 4°C for 3 min). The cells were fixed for 20 min in 1 ml 1% paraformaldehyde (in PBS).

Flow cytometric analysis of the paraformaldehyde fixed cells was carried out by FACScan flow cytometer with an excitation wavelength of 488 nm. R123 and propidium iodate fluorescence were determined for 15 000 cells, considering only viable cells (PI-excluding cells). Fluorescence data were shown on a three-decade log scale.

Data analysis

All cytotoxicity data were modelled by non-linear regression using Kaleidagraph (Ablebeck Software, USA). Dose–response curves for cytotoxicity of drugs in absence or presence of the P-gp inhibitor GF120918 were analysed by non-linear curve fitting, using measured absorption of formazan produced by viable cells at 550 nm wavelength. Data were modelled with the general dose–response equation (DeLean et al, 1981):

\[ Y = \left( \frac{a - d}{1 + \left( \frac{X}{C} \right)^b} \right) + d \]

where \( Y \) is the absorption of formazan salts produced by viable cells at a molar concentration of cytotoxic drug \( X \). The maximum of the curve is \( a \), the minimum is \( d \), the slope factor is \( b \) and \( C \) the drug concentration which inhibits the cell proliferation by 50%. For most curves \( a, b, c \) and \( d \) were the modelled parameters.

The effect of GF120918 to sensitize MES-Dx5 cells to cytotoxic drugs was expressed as % maximal shift of resistance, using the following formula:

\[ \frac{EC_{50R} + EC_{50I}}{EC_{50R} + EC_{50I}} \times 100 \]

with the \( EC_{50} \) of the cytotoxic drug required to inhibit cell proliferation by 50% for \( R \) the resistant cell line MES-Dx5, \( S \) the sensitive cell line MES-SA and \( I \) the P-gp inhibitor GF120918 at various concentrations. Ligand binding experiments were analysed by non-linear curve fitting as previously described (Malkhandi et al, 1995).

Means from independent experiments (\( n \)) are given with standard errors of mean, s.e.m. Statistical significance was analysed with the \( t \)-test.

RESULTS

Reversal of MES-Dx5 resistance to cytotoxics by GF120918

The wild-type MES-SA cells were subjected to a 3-day exposure to various cytotoxic agents and cell viability was assessed with the formazan-based MTT assay. The MES-SA cells were sensitive to vinblastine, taxotere and taxol with an \( EC_{50} \) of 3 ± 1 nM (\( n = 4 \)), 5 ± 1 nM (\( n = 2 \)) and 5 ± 1 nM (\( n = 5 \)) respectively, less sensitive to doxorubicin with an \( EC_{50} \) of 58 ± 14 nM (\( n = 5 \)) and etoposide with an \( EC_{50} \) of 3003 ± 106 nM (\( n = 5 \)) (Table 1 and Figure 2 A–E).

The doxorubicin selected cell line MES-Dx5 exhibited the following resistance pattern compared to the parental cell line MES-SA: taxol 94-fold, taxotere 19-fold, vinblastine tenfold, doxorubicin ninefold and etoposide twofold resistance (Table 1 and Figure 2 A–E). One striking feature of these dose–response curves is the variation in the steepness of the slope, a topic usually ignored in the literature.

Table 1 \( EC_{50} \) (nM) of cytotoxic drugs in MES-SA and MES-Dx5 cells (MTT dye-based cytotoxicity assay after 72-h exposure to drugs) and resistance factor for MES-Dx5 cells.

| Drug          | MES-SA          | MES-Dx5         |
|---------------|-----------------|-----------------|
|               | \( EC_{50} \) (nM) | Slope | \( n \) | RF* |
| Etoposide     | 3003 ± 1006     | 0.4 ± 0.1       | 5    |     |
| Doxorubicin   | 58 ± 14         | 0.9 ± 0.2       | 5    |     |
| Vinblastine   | 3 ± 1           | 1.6 ± 0.3       | 4    |     |
| Taxotere      | 5 ± 0.1         | 2.6 ± 0.1       | 2    |     |
| Taxol         | 5 ± 0.1         | 2.0 ± 0.2       | 5    |     |
| Doxorubicin   | 6846 ± 504      | 1.0 ± 0.2       | 3    | 2   |
| Vinblastine   | 534 ± 67        | 0.9 ± 0.1       | 13   | 9   |
| Taxotere      | 30 ± 12         | 0.6 ± 0.1       | 6    | 10  |
| Taxol         | 93 ± 28         | 0.5 ± 0.1       | 2    | 19  |
| Etoposide     | 470 ± 98        | 0.5 ± 0.1       | 8    | 94  |

*Resistance factor; \( ^{+}P = 0.0003 \) slope MES-Dx5 vs MES-SA cells; \( ^{–}P < 0.0001 \) slope MES-Dx5 vs MES-SA cells.
In MES-Dx5 cells a low slope was observed for the dose–
response curve of all tubulin inhibitors (vinblastine < taxol <
taxotere) (Table 1) compared to (i) doxorubicin and etoposide
or (ii) the dose–response curves in MES-SA cells. The
dose–response curves for taxanes in particular had slope factors of
< 1.0 (P < 0.0001), implying that proportional increases in the
doses of taxanes have less effect than for drugs with steep
dose–response curves such as doxorubicin.

The addition of the P-gp inhibitor GF120918 (100 nM ) for 72 h
did not affect sensitivity of the parental MES-SA cells to any of
the five cytotoxics tested (results not shown). GF120918 alone did
not produce a cytotoxic effect during the 72-h exposure in the
MTT assay. An example of the sensitizing effect of GF120918 for
taxol in MES-Dx5 cells is shown in Figure 3A. GF120918 concen-
trations of greater than 10 nM steepened the dose–response curves
for taxol, vinblastine and taxotere. GF120918 was able to fully
reverse resistance of the MES-Dx5 cells to the level of drug sensi-
tivity shown by the parental MES-SA cells for all tested cytotoxic
drugs. Based upon the analysis undertaken, the EC50 of GF120918
to sensitize MES-Dx5 cells to taxol was 91 – 23 nM (n = 8) but
7 – 2 nM (n = 3) for etoposide (Table 2, Figure 3 B, C). The
relative resistance to each cytotoxic agent correlated with the
GF120918 concentration needed to restore 50% of sensitivity ($r = 0.9$, $P = 0.03$). To test if this observation is due to P-gp in MES-Dx5 cells or the effect of GF120918, we have performed reversal experiments with another P-gp inhibitor, verapamil, in MES-Dx5 cells which modulated resistance to taxol, doxorubicin, vinblastine and etoposide equipotently with an EC$_{50}$ of ~3 μM (Figure 3D).

### [3H]-vinblastine, [3H]-taxol and [14C]-doxorubicin accumulation assays

The non-P-gp expressing MES-SA cells accumulated [3H]-vinblastine, [3H]-taxol and [14C]-doxorubicin in a time-dependent manner. MES-Dx5 cells accumulated radiolabelled drugs with a similar time course, but accumulated 20% less [3H]-taxol, 20% less [14C]-doxorubicin and 35% less [3H]-vinblastine at steady state. GF120918 did not increase the cellular content of
radiolabelled cytotoxics in the sensitive MES-SA cells (data not shown). However in MES-Dx5 cells, at a saturating concentration of 1 μM, GF120918 increased the cellular accumulation of all three drugs by 30% at 4 h. In MCF-7/ADR cells, a breast cancer cell line selected in doxorubicin with sixfold higher expression of P-gp, 1 μM GF120918 increased the uptake of [14C]-doxorubicin by 240% (data not shown).

A dose-dependent effect of GF120918 existed for all three radiolabelled cytotoxic drugs (Figure 4). The EC50 for the reversal of the accumulation deficit by GF120918 in MES-Dx5 cells ranged from 37–64 nM (Table 2).

**Gel electrophoresis and Western blotting of membrane preparations**

The monoclonal antibody NCL-JSB-1 could detect P-gp in Western blots of membranes prepared from MES-Dx5 cells (limit of detectability 10 μg membrane protein) but did not in MES-SA membranes. The calculated molecular weight for P-gp in MES-Dx5 cells was 156 kDa (data not shown).

**FACS analysis of P-gp expression**

FACS analysis of rhodamine accumulation and its efflux was used to determine if the MES-Dx5 cells consisted of more than one population. MES-Dx5 cells were found to consist of a single-cell population with regard to rhodamine efflux (Figure 5).

**Membrane binding assay**

Saturation analysis of [3H]-vinblastine binding to MES-Dx5 cell membrane preparations revealed a Bmax of 5.2 ± 1.6 pmol mg–1 protein and a Kd of 6.8 ± 1.4 nM. The addition of ATP and MgCl2 had only a minor effect on [3H]-vinblastine binding (Figure 6).

[3H]-vinblastine binding to membrane preparations of MES-SA was not displaceable by cyclosporin A (Figure 7A), a well described, competitive inhibitor of [3H]-vinblastine binding to P-gp (Ferry et al, 1992). Cyclosporin A inhibited [3H]-vinblastine binding to P-gp in MES-Dx5 membrane preparations proportional to the amount of protein used (up to 300 μg). Cyclosporin A inhibited [3H]-vinblastine binding to P-gp to the level of non-specific binding in MES-SA cells.

Inhibition of [3H]-vinblastine binding to MES-Dx5 membranes was assessed using several cytotoxic agents and P-gp inhibitors. Doxorubicin was a weak inhibitor with a Ki = 6940 ± 2100 nM followed in increasing potency by verapamil (Ki = 660 ± 350 nM), tarox (Ki = 400 ± 135 nM) and GF120918 (Ki = 5 ± 1 nM) (Figure 7B). Taxol, an allosteric inhibitor of [3H]-vinblastine binding (Ferry et al, 1994), could displace only 40% of [3H]-vinblastine bound to P-gp.
Effect of a high concentration of fetal calf serum on the potency of GF120918 in MCF-7/ADR cells

MES-Dx5 cells grew poorly in medium supplemented with 50% FCS. Therefore MCF-7/ADR cells and their drug-sensitive wild-type MCF-7 cells were used to explore the effect of a high protein concentration on the potency of GF120918 (MTT assay). The cells were incubated with the cytotoxic drug taxol in the presence of a series of potent P-gp inhibitors and either 10% or 50% FCS. The data are shown in Table 3. Although all P-gp inhibitors were less potent in 50% FCS, this effect was significantly smaller ($P < 0.05$, t-test) for GF120918 (2.6–1.5-fold) compared to CP100356 (88–22-fold), dexniguldipine (37–9-fold), CGP 412251 (30–12-fold) or PSC833 (19–6-fold).

**DISCUSSION**

MES-Dx5 cells were derived from a human uterine sarcoma cell line, MES-SA after long-term selection in 500 nM doxorubicin (Harker et al, 1983; Harker and Sikic, 1985). The resistance factors to doxorubicin, vinblastine and etoposide in the 72-h MTT cytotoxicity assay we report were lower than those previously reported for the 1-h drug exposure in a colony-forming assay (Harker and Sikic, 1985). This is not surprising for tubulin-binding drugs and etoposide which show marked cell-cycle/phase dependency. After a 72-h exposure of MES-Dx5 cells to cytotoxic drugs, a resistance pattern similar to the one we have observed has been described (Gosland et al, 1989).

MES-Dx5 cells expressed P-gp as assessed by Western blotting and [3H]-vinblastine binding, which allowed quantification of the number of P-gp molecules per mg protein. In the drug sensitive clone MES-SA P-gp could not be detected by either method. The density of [3H]-vinblastine binding sites in MES-Dx5 membrane preparations was about 25% that of MCF-7/ADR cells (Ferry et al, 1995). This correlates with the higher resistance of MCF-7/ADR cells to e.g. doxorubicin (200-fold) and taxol (1000-fold) (MA Russell and DR Ferry, unpublished data).
One striking feature of the dose–response curves of the cytotoxic drugs in MES-Dx5 cells was the variation in their slopes. Thus the slopes of the dose–response curves for taxanes and vinblastine were significantly lower than for doxorubicin. When the P-gp inhibitor GF120918 (Hyafil et al, 1993), an acridone-carboxamide, was added to these cells, the slopes of the dose–response curves for taxanes and vinblastine became steeper, reaching the same level as those observed in the parental MES-SA cells.

One possible explanation for this phenomenon could be, that MES-Dx5 cells consisted of two or more populations of cells expressing different amounts of P-gp. However, in a rhodamine FACS analysis (using the method described by Davies et al (1996)), a single population of MES-Dx5 cells was detected. Other explanations needed to be further considered, including heterogeneity of tubulin expression due to mutations in microtubules. Mutations in tubulin subunits were described in a series of mutant Chinese hamster ovary cell lines which rendered the cells resistant to taxol (Schibler and Cabral, 1986). Assuming the level of resistance to taxol in MES-Dx5 cells was due to the combined effects of P-gp and mutated microtubulin, the inhibition of P-gp should only lead to a shift of the dose–response curve to the left, e.g. concentrations of cytotoxic drugs achieve higher level of cytotoxicity but not to an alteration in the steepness of the dose–response curve. This was clearly not the case since the P-gp inhibitor GF120918 fully sensitized MES-Dx5 and additionally increased the slope of the dose–response curve for all tubulin binding drugs, taxol, taxotere and vinblastine.

MES-Dx5 cells were fully sensitized to a variety of natural product cytotoxics with nM concentrations of the recently described potent P-gp inhibitor GF120918, achieving the same sensitivity as the parental MES-SA cells. GF120918 acted potently on P-gp with concentrations as low as 1 nM, exhibiting a measurable reversal of resistance in the MTT assay. However, fivefold higher concentrations of GF120918 were required to reverse resistance to taxol by 50% compared to doxorubicin and vinblastine. Similarly an eightfold higher concentration of GF120918 was required to reverse resistance to taxotere by 50% compared to etoposide. If GF120918 bound to a common site, ‘plugging’ P-gp, the EC_{50} to reverse resistance expressed in the cytotoxicity assay and the EC_{m} to increase the cellular accumulation of drugs, should be equal. A number of possible explanations for this phenomenon are discussed below.

The potency of GF120918 to reverse resistance relates to the affinity of cytotoxics to bind to P-gp

We know that [\(^{3}H\)]-GF120918 can bind saturably and with a 0.8 nM \(K_{D}\) to human P-gp (Ferry et al, 1996). Theoretically the explanation may lie in the relative affinities of the cytotoxic drugs for P-gp. Thus, if drug A (e.g. taxol or vinblastine) has a very high affinity for P-gp relative to drug B (e.g. doxorubicin or etoposide) and both are present at equal concentrations, more GF120918 will be needed to inhibit the binding of drug A by 50%. However, there was no clear relationship between the potency to modulate a given cytotoxic drug and the affinity to bind to P-gp. Furthermore, since the EC_{50} for GF120918 to reverse drug resistance was higher for taxol than for vinblastine this explanation must be flawed. These observations therefore imply that P-gp cannot be regarded as a simple receptor at which drugs compete.

The resistance factor correlates with the concentration of GF120918 required to modulate the resistance

The striking correlation between the EC_{50} of GF120918 to reverse resistance in the cytotoxicity assay and the resistance factor of each cytotoxic agent (\(r = 0.9\)) suggests that the potency of GF120918 relates closely with the resistance factor of the cytotoxic drug. This implies, that a given concentration of GF120918 induces a given fractional modulation of resistance. It is difficult to envisage how this could occur at the molecular level.

Although there is no direct evidence, it is mechanistically plausible that GF120918 is a more potent modulator of resistance to vinblastine, doxorubicin and etoposide than the taxanes probably as a consequence of the pattern of binding to P-gp and subsequent inhibition of substrate transport by P-gp in MES-Dx5 cells. Experiments aimed at measuring direct binding of [\(^{3}H\)]-taxol and...
[1H]-GF120918 to P-gp may cast some light and give some clues as to the molecular mechanisms underlying the phenomenon observed in the cytoxicity assays. GF120918 is the most potent P-gp inhibitor described (Hyafil et al, 1993) and retains most of its activity at high protein concentrations (50% FCS). All other tested P-gp inhibitors, i.e. CP100356, dexniguldipine, CGP 412251 and PSC833 were significantly less potent in the presence of 50% FCS. This finding is important for clinical studies in view of the high protein concentrations present in whole blood. In this manuscript detailed pharmacological data regarding the reversal of resistance to vinblastine, doxorubicin, etoposide, taxotere and taxol by GF120918 have been documented for the first time. The results have significant implications on a number of levels. Functional studies of P-gp, particularly binding and transporting of cytotoxic agents and P-gp inhibitors are not yet sophisticated enough to estimate initial rates of transport or turnover rates for any substrate. The relatively low expression of P-gp in cells was a major problem which some groups recently overcame by purifying P-gp (Shapiro et al, 1995; Callaghan et al, 1997). This may allow to re-address these fundamental questions in the future.

GF120918 has entered phase I clinical trials in combination with doxorubicin (Ferry et al, 1998; Planting et al, 1998). The serum levels achieved are known to reverse the P-gp mediated efflux of rhodamine in CD56 cells (natural killer cells) in normal volunteers (Kerr et al, 1996) and the full trial data will be presented soon (Ferry et al, in preparation).

In terms of the future clinical application, it may prove important that the resistance to doxorubicin could be modulated at lower concentrations of GF120918 compared to the taxanes, taxol and taxotere but remains to be tested.

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REFERENCES

Barrand MA, Heppel-Parton AC, Wright KA, Rabbits PH and Twentyman PR (1994) A 190k protein overexpressed in non-P-glycoprotein containing MDR cells and its relation to the MRP gene. J Natl Cancer Inst 86: 110–117

Boer R, Dichié M, Borechers C, Ulrich WR, Marecek JF, Prestwich GD, Glossmann H and Streising J (1996) Reversible labeling of a chemosensitizer binding domain of P-glycoprotein with a novel 1,4-dihydropyridine drug transport inhibitor. Biochemistry 35: 1387–1396

Callaghan R, Bertridge G, Ferry DR and Higgins CF (1997) The functional purification of P-glycoprotein is dependent on maintenance of a lipid protein interface. Biochem Biophys Acta Biomembranes 1328: 109–124

Chen G, Jafferzoi JP, Fleming WH, Duran GE and Sicik BI (1994) Prevalence of multidrug resistance related to activation of the mdr1 gene in human carcinoma mutants derived by single-step doxorubicin selection. Cancer Res 54: 4980–4987

Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz E, Duncan AMV and Deely RG (1992) Over-expression of a transporter gene in a multidrug resistant human lung cancer cell line. Science 258: 1650–1653

Davies R, Budworth J, Riley J, Snowden R, Gescher A and Gani T (1996) Regulation of P-glycoprotein 1 and 2 gene expression and protein activity in two MCF-7/Dox cell line subclones. Br J Cancer 73: 307–315

Day S, Ramachandra M, Pastan I, Gottesman MM and Ambudkar SV (1997) Evidence for two non-identical drug-interaction sites in the human P-glycoprotein. Proc Natl Acad Sci USA 94: 10594–10599

DeLean A, Hancock AA and LeFkowitz RJ (1981) Validation and statistical analysis of a computer modelling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. Mol Pharmacol 21: 5–16

Endicott JA and Ling V (1989) The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem 58: 137–171

Ferry DR, Russell MA and Cullen MH (1992) P-glycoprotein possesses a 1,4-dihydropyridine-selective drug acceptor site which is allosterically coupled to a vinca alkaloid-selective binding site. Biochem Biophys Res Commun 188: 440–445

Ferry DR, Russell MA and Kerr DJ (1994) [1H]-Taxol binds to a drug acceptor site which is allosterically coupled to the vinblastine-selective site of P-gp. Proc Am Assoc Cancer Res 35: 348

Ferry DR, Malkhandi PJ, Russell MA and Kerr DJ (1995a) Allosteric modulation of [1H]-vinblastine binding to P-glycoprotein of MCF-7 ADR breast cancer cell membranes. Biochem Pharmacol 49: 1851–1861

Ferry DR, Malkhandi PJ, Russell MA and Kerr DJ (1995b) Allosteric regulation of [1H]-vinblastine binding to P-glycoprotein of MCF-7 ADR cells by dexniguldipine. Biochem Pharmacol 49: 1851–1861

Ferry DR, Russell MA, Kerr DJ, Correa JD and Prakash SR (1996) [1H]-GF918 (GF120918) binds with positive co-operativity to human P-glycoprotein with a nM dissociation constant. Proc Am Assoc Cancer Res 37: abstract 2246

Ferry D, Moore M, Bartlett NL, Fyle D, Oza G, Fracasso PM, Kersey W, Kessel PS, Jewell RC and Paul EM (1998) Phase I and pharmacokinetic (PK) study targeting a 500 ng/ml plasma concentration of the potent multidrug resistance (MDR) modulator GF120918 (GF) with doxorubicin (DOX) in patients with advanced solid tumors. Proc Am Soc Clin Oncol 17: 24a0

Ford JM and Hain WN (1990) Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol Rev 42(3): 155–199

Gosland MP, Lum BL and Sicik BI (1989) Reversal by cefoperazone of resistance to etoposide, doxorubicin, and vinblastine in multidrug resistant human sarcoma line. Cancer Res 49: 6901–6905

Gottesman MM and Pastan P (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62: 385–427

Harker WG and Sicik BI (1985) Multidrug (pleiotropic) resistance in doxorubicin selected variants of the human sarcoma cell line MES-SA. Cancer Res 45: 4091–4096

Harker WG, MacIntosh FR and Sicik BI (1983) Development and characterization of a human sarcoma cell line, MES-SA, sensitive to multiple drugs. Cancer Res 43: 4943–4950

Hyafil F, Vergely C, Du VP and Grand PT (1993) In vitro and in vivo reversal of multidrug resistance by GF120918, an acrideroncarboxamide derivative. Cancer Res 53(19): 4595–4602

Kajji S, Dresslin JA, Grizzuti K and Gross P (1994) Structurally distinct MDR modulators show specific pattern of reversal against P-glycoproteins bearing unique mutations at serine (939/941). Biochem 33(17): 5041–5048

Malkhandi PJ, Ferry DR, Boer R and Kerr DJ (1995) P-glycoprotein has a drug acceptor site for 1,4-dihydropyridines which is localised on an intracellular domain. Proc Am Assoc Cancer Res 36: 332

Miller TP, Grogan TM, Dalton WS, Spier CM, Scheper RJ and Salmon SE (1991) P-glycoprotein expression in malignant lymphoma and reversal of clinical drug resistance with chemotherapy plus high dose verapamil. J Clin Oncol 9: 17–24

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63

Planting A, van der Gaast A, Sparreboom A, van der Burg MEL, de Boer M, Wissel PS, Jewell RC, Paul EM and Verweij J (1998) Phase I and pharmacokinetic (PK) study targeting a 100 ng/ml plasma concentration of the potent multidrug resistance (MDR) modulator GF120918 (GF) with doxorubicin (DOX) in patients with advanced solid tumors. Proc Am Soc Clin Oncol 17: 199a

Raviv Y, Poland HB, Bruggemann EP, Pastan I and Gottesman MM (1990) Photosensitising labeling of a functional multidrug transporter in living drug resistant tumour cells. J Biol Chem 265: 3975–3980

Sambrook J, Fritsch EF and Maniatis T (1989). Transfer of proteins from SDS-polyacrylamide gels to solid supports: immunological detection of
immobilized proteins (Western blotting). In: Laboratory Cloning, a Laboratory Manual, Nolan C (ed), pp. 18.60–18.75. Cold Spring Harbor Laboratory Press: New York
Scheper RJ, Broxterman HJ, Scheffer GL, Kaaijk P, Dalton WS, van Heijningen THM, van Kalken CK, Slovak ML, de Vries EGE, van der Valk P, Meijer CJLM and Pinedo HM (1993) Overexpression of a M, 110 000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res 53: 1475–1479
Schibler MJ and Cabral F (1986) Taxol-dependent mutants of chinese hamster ovary cells with alterations in alpha-tubulin and beta-tubulin. J Cell Biol 102: 1522–1531
Shapiro AB, Ling V and Doige CA (1995) Reconstitution of drug transport by purified P-glycoprotein. J Biol Chem 270: 16167–16175
Sonneveld P, Durie BGM, Lokhorst HM, Marie J-P, Solbu G, Zittoun R, Lowenberg B and Nooter K (1992) Modulation of multidrug resistant myeloma by cyclosporin. Lancet 340: 255–258
Spoelstra EC, Westerhoff HV, Dekker H and Lankelma J (1992) Kinetics of daunorubicin transport by P-glycoprotein of intact cells. Eur J Biochem 207: 567–579
Witherspoon SM, Emerson DL, Kerr BM, Lloyd TL, Dalton WS and Wissel PS (1996) Flow cytometric assay of modulation of P-glycoprotein function in whole blood by the multidrug resistance inhibitor GG918. Clin Cancer Res 2: 7–12