**In Vitro and in Vivo Reversal of Multidrug Resistance by GF120918, an Acridonecarboxamide Derivative**

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**ABSTRACT**

N-4-[(2,3,4-tetrahydro-6,7-dimethoxy-2-isquinolinyl)-ethyl]-phenyl-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918) has been selected from a chemical program aimed at identifying an optimized inhibitor of multidrug resistance (MDR). The potency of GF120918 is assessed by dose-dependent sensitization of CHO/5S, OVI/DXR and MCF7/ADR cells to the cytotoxicity of doxorubicin and vincristine respectively: GF120918 fully reverses multidrug resistance at 0.05 to 0.1 μM and is half maximally active at 0.02 μM. The spectrum of drugs sensitized by GF120918 coincides with those having the classical MD phenotype. In CHO/5S cells, 0.01–0.1 μM GF120918 enhances the uptake of [3H]dauorubicin and blocks the efflux from preloaded cells. It is also shown that GF120918 is still active several hours after being taken away from the culture medium showing that it is not, like verapamil, effluxed rapidly by P-glycoprotein. GF120918 effectively competes with [3H]azidopine for binding P-glycoprotein, pointing to this transport membrane protein as its likely site of action.

After i.v. administration to mice, GF120918 penetrates thoroughly various organs that have a tissue level/blood level ratio above 10. It is eliminated from organs and blood with a half-time of approximately 2.7 h. It is well absorbed after p.o. administration.

In mice implanted i.p. with the MDR P388/Dox tumor, a single i.v. or p.o. dose of GF120918 restores sensitivity of the tumor to a single i.p. dose (5 mg/kg) of doxorubicin administered 1 h later. A statistically significant effect is observed at 1 mg/kg GF120918 i.v. and maximal effect is reached at 5 mg/kg. Similarly, whereas neither drug alone is effective, GF120918 (10 mg/kg i.p.) associated with doxorubicin (5 mg/kg i.p.) inhibits the growth of the moderately MDR C26 tumor implanted s.c. as assessed by tumor size at day 19. GF120918 does not modify significantly the distribution or the elimination of doxorubicin in mice ruling out the possibility that the antitumor effects seen might be explained by pharmacokinetic interactions.

**INTRODUCTION**

The MDR2 phenotype has been well characterized since the mid-1980s: cross-resistance to unrelated classes of cytotoxics (called thereafter MDR cytotoxics) is linked to the increased expression of a cell surface pump now known as P-glycoprotein; reversal of MDR can be obtained by treatment with numerous classes of lipophilic positively charged drugs known as MDR inhibitors (for reviews, see Refs. 1–3). Later studies have now substantiated the importance of the MDR phenotype in the acquired or intrinsic resistance of human tumors to chemotherapy (4, 5) and raised hopes that addition of MDR inhibitors to cytotoxics might improve chemotherapy success rate. Clinical Phase I and Phase II studies have been thus far disappointing, often because of the limited tolerance of prototype MDR inhibitors by themselves, which precluded attainment of potentially active levels in patients (2, 5). For example, full reversion of MDR by verapamil requires an approximate 10 μM concentration in most cell culture models whereas plasma levels above 1 μM result in atrioventricular blocks in patients (6).

The design of potent MDR inhibitors devoid of other pharmacological activities has thus become a desirable goal to test the MDR reversal hypothesis in the clinics. In the course of a chemical program aimed at identifying potent MDR inhibitors, we have identified a series of acridone carboxamide derivatives (7). We describe here the properties of one of them, GF120918 (Fig. 1).

In vitro, GF120918 is consistently active at concentrations around 20 nm in a series of cellular models for MDR inhibition: sensitization of MDR cells to cytotoxics, increased anthracycline uptake, and inhibition of efflux. GF120918 binds to P-glycoprotein as witnessed by inhibition of [3H]azidopine photoaffinity labeling.

In two animal models of MDR, the P388/Dox leukemia and the C26 colon carcinoma, a single i.v. or p.o. dose of GF120918 restores the antiproliferative action of doxorubicin.

**MATERIALS AND METHODS**

**Cell Lines**

The MDR Chinese hamster ovary cell line CHO/5S selected in vitro against colchicine (8,9) was provided by Dr. V. Ling. It was maintained as anchorage-dependent monolayers in a minimum essential medium supplemented with thymidine, adenine, 10% fetal bovine serum, 2 mM L-glutamine (Flow), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2. Cells were passaged into Falcon culture flasks twice/week after dissociation with trypsin-EDTA.

The human breast cancer doxorubicin-selected variant cell line (10) (MCF7/ ADR) was produced by Dr. K. Cowan. It was maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2. Cells were split once a week after dissociation with trypsin-EDTA. For photoaffinity radiolabeling experiments, expression of P-glycoprotein was boosted by 2 weeks of culture in the presence of increasing concentration of rhodamine 123 up to 0.2 μM (11).

The ovarian carcinoma doxorubicin-selected cell line (12) (OVI/ DXR) was obtained from Dr. J. Benard (Institut Gustave Roussy, Villejuif, France) and was cultured as MCF7/ADR.

The C26 colon carcinoma cells line was obtained from B. Chauvet (IN- SERM U252, Dijon, France). Cells were maintained in Dulbecco's modified Eagle's medium containing 15% fetal calf serum and split after dissociation by gentle aspiration.

The P388 and P388/Dox mouse leukemia cell lines were obtained from Dr. G. Atassi (Institute J. Bordet, Brussels, Belgium). They were maintained for up to 30–35 passages by weekly i.p. passage in DBA/2 female mice (IFFA CREDO, l'Arbresle, France). For in vitro sensitivity assays, ascites cells were collected and cultured for up to 30 days in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum.

The PE117 lymphoblastoid cell line was from Dr. L. Degos (Hôpital Saint Louis, Paris, France). Other cell lines were from the American Type Culture Collection.

**Drugs**

For cell culture studies GF120918 solutions were prepared as follows: a 2.5 mM solution was prepared in methanol/HCl 0.1 M (4:1). This stock solution was diluted 1:1000 in complete culture medium. We have noticed that solutions for cell culture studies should always be prepared by diluting the stock solution in

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1To whom requests for reprints should be addressed.

2The abbreviations used are: MDR, multidrug resistance; GF120918, N-4-[(2,1,2,3,4-tetrahydro-6,7-dimethoxy-2-isquinolinyl)-ethyl]-phenyl-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; dox, doxorubicin; LD50, 50% lethal dose; IC50, concentration producing 50% inhibition.
medium containing 5% fetal calf serum or equivalent amounts of protein.

The following drugs were dissolved as GF120918: gramicidin D; colchicine; verapamil hydrochloride; quinidine (all from Sigma).

The following products were dissolved directly in culture medium: doxorubicin chloride (Adriblastine; Farmitalia Carlo Erba, Rueil, France); amiodarone chloride (Cordarone, for injection; Sanofi Winthrop, Gentilly, France); vincristine sulfate (Oncovin; Lilly); vinblastine sulfate (Velbe; Lilly); amiodarone chloride (Cordarone, for injection; Sanofi Winthrop, Gentilly, France); vincristine sulfate (Oncovin; Lilly); vinblastine sulfate (Velbe; Lilly); aclarubicine chlorhydrate (Aclacinomycine; Roger Bellon, France); mitoxantrone chlorhydrate (Novantrone; Lederle); etoposide (Vepeside; Sanofi Winthrop); bleomycin (Roger Bellon); cyclosporin A (Sandimmun; Sandoz).

For photoaffinity radiolabeling experiments, GF120918 was dissolved as a 10 mM stock solution in dimethylsulfoxide and then diluted immediately before use in 0.2 mM HCl.

For in vivo studies, GF120918 is prepared as a 10 mg/ml stock solution of the HCl salt in 1.2 propanediol/H2O (G/3, v/v). Immediately before the assay, the stock solution is diluted in H2O to the desired concentration.

Drug Sensitivity Assays

CHP5 cells were seeded at a density of 10^4 cells/well in Falcon microtiter plates. After 24 h, the medium was removed and replaced by 0.1 ml of fresh medium containing MDR inhibitors. A 0.1 ml volume of 2-fold dilution of doxorubicin was added. After 72 h incubation, cell viability was assayed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) to a dark blue formazan product (13, 14). Briefly 20 μl of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate-buffered saline were added to each well. After 4 h incubation at 37°C medium was aspirated and replaced by 0.1 ml dimethylsulfoxide. After vigorous shaking the quantity of formazan product formed was assessed by its absorbance at 550 nm on a Dynatech MR 700. The average of duplicate wells was used for the calculation. A LDA2 for doxorubicin was then derived in the absence or in the presence of various MDR inhibitors. To study the spectrum of drugs potentized by GF120918, similar assays were run with a variety of cytotoxins ± a fixed concentration of 50 nM GF120918.

Drug sensitivity assays on OVI/DXR, C26, P388/Dox were carried out as for CHP5 cells except for a 48 h culture between seeding and test (OVI/DXR) or the use of round bottom plates and the introduction of centrifugation steps for washing procedures for P388/Dox.

For the MCF7/ADR assay, a range of vinblastine concentration was used on cells seeded at 2 x 10^5/well. Moreover, viability at the end of the assay was assessed by a methylene blue staining technique. Briefly, at the end of 72 h incubation, the supernatants were aspirated and the cell monolayer was washed with phosphate-buffered saline and fixed for 15 min with 0.1 ml methylene blue (Sigma) at 0.1% dissolved in distilled H2O. The plates were washed extensively under running water and then dried at 56°C for 15 min. The dye was eluted by shaking with 0.1 ml of HCl 0.1 M and absorbances were read at 630 nm.

Uptake of [3H]Doxorubicin by CHP5 Cells

Four x 10^4 cells/well were seeded into a 96-well microtiter plate. Twenty-four h later, the medium was aspirated and replaced by 0.05 ml of culture medium with 10% fetal calf serum containing increasing concentrations of MDR inhibitors. [3H]Doxorubicin (85 GBq/mmoll (New England Nuclear) was added as a 0.05 ml solution in culture medium + serum to a final concentration of 0.7 μM.

After a 4-h incubation under culture condition, supernatants were thoroughly aspirated and the radioactive material incorporated into cells is eluted overnight at 4°C by 0.1 ml NaOH, 0.1 M.

[3H]Doxorubicin Efflux from CHP5 Cells

Twenty-four h before the assay 10^5 cells/well were seeded into 24-well culture plates (Falcon). For the assay, cells are loaded with [3H]Doxorubicin in 0.5 ml culture medium deprived of glucose and containing 10 mM NaCl, 0.28 μM [3H]Doxorubicin with 5% dialysed fetal calf serum with or without MDR inhibitors. After 2 h of incubation, cells were washed twice rapidly (-10 s for each wash) with efflux medium (see below) and 0.5 ml of this medium was added to the well at time 0 of the kinetic. Efflux medium was normal culture medium buffered at pH 7.8 by 10 mM NaHCO3 and 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid with or without MDR inhibitors.

The kinetics of efflux was assayed at 20°C in ambient air. Quantitation of [3H]Doxorubicin effluxed from cells was determined by harvesting the culture medium at each assay time and replacing it with fresh medium with or without MDR inhibitors.

At the end of the kinetics, cells were lysed in 0.1 M NaOH to assess remaining intracellular doxorubicin. Total incorporated [3H]Doxorubicin was calculated from the sum of [3H]Doxorubicin effluxed during the assay and that left in the cells at the end. Results are expressed as the percentage of [3H]Doxorubicin remaining in cells/totai incorporated doxorubicin.

Photoaffinity Radiolabeling of P-glycoprotein with [3H]Azidopine

CHP5 cell monolayers were washed twice with phosphate-buffered saline, scraped, and centrifuged 5 min at 1500 rpm. The cell pellet was resuspended in 10 mM Tris, 2 mM EDTA, 2 mM ethyleneglycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (pH 7) and maintained in ice for membrane preparation. Membrane vesicles were prepared by sonication the cell suspension with Vibra Cell VC40 and ASI sound 4 times for 1 s at 20 kHz. Nucleus and cell debris were eliminated by centrifugation at 1500 g 15 min and the membrane suspension was pelleted by centrifugation for 30 min at 100 000 g. This membrane enriched fraction was resuspended in the same buffer and stored at -80°C with 10% of glycerol.

Ten μl of unlabeled cell membrane suspension (at 0.4 mg of protein/ml) were aliquoted in to each well in a V-shaped 96-well plates. Five μl of GF120918 were then added to each well. The plate was incubated 25 min at 25°C in the dark. Then, 5 μl of tritiated azidopine (1.8 TBq/mmoll, Amersham) (0.6 μM in HCl 0.2 mM) were added to each well. After 25 min of incubation at 25°C in the dark, samples were simultaneously irradiated for 2 min at 254 nm at 0°C with a thin layer chromatography-designed UV lamp (Bioblock, Illkirch, France) directly in contact with the plate. Samples were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer but not heated because of described aggregation of P-glycoprotein at high temperature even in sample buffer (15). After separation on a 7.5% polyacrylamide gel, the gel was treated for fluorography with Amplify (Amersham) and exposed during 3 days onto a photosensitive film (Hyperfilm MP, Amersham). The fluorography was analysed using a Camag thin layer chromatography Scanner II densitometer. Western blot identification of P-glycoprotein was performed using polyclonal rabbit antiserum (mdr Ab-1; Oncogene Sciences) and enhanced chemiluminescence Western blot detection kit (Amersham).

Animal Models

P388/Dox. Female C57BL/6 × DBA/2 F1 mice (IFFA CREDO) weighing 20–25 g were randomized by groups of 10 and acclimated for at least 4 days before the test. On day 0, P388/Dox ascites from DBA/2 mice used for passages were pooled and adjusted to 10^6 cells/ml and a 0.1 ml inoculum was injected i.p. to each mouse.

GF120918 was administrated on day 1 i.v. in the retro-orbital vein, or by gavage. One h later, a 5 mg/kg solution of doxorubicin was injected i.p. under a 0.1 ml volume.
Mortality was observed daily until day 30. Data were subjected to statistical interpretation using the Kaplan-Meier method, log rank test, by Dr. R. Caillon (Medical Statistics; Glaxo, Paris, France).

C26 colon carcinoma cells were resuspended at a concentration of 10^6 cells/ml. On day 0, a 0.1 ml solution was injected s.c. to female BALB/c mice weighing 18–20 g. On day 1, 0.1 ml of a GF120918 solution was injected i.p. One h later, a 5 mg/kg solution of doxorubicin was injected i.p. under a 0.1 ml volume.

Tumor measurements were made with a caliper twice/week until day 16 to 19. Tumor volumes were calculated by the formula 1/2 × L × l × F where L is the larger diameter and l the smaller diameter of the tumor. Results are interpreted according to the F test.

**Pharmacokinetics of GF120918.** Female mice (C57BL/6 × DBA/2 F1) received 10 mg/kg (i.v. route) or 20 mg/kg (p.o. route). At times 0.25, 0.5, 1, 2, 4, 6 h, (three) animals were sacrificed. Blood, heart, liver, lungs, brain, and kidneys were analyzed. For each organ, GF120918 was extracted at pH 9 by diethyl ether and assayed by the following high pressure liquid chromatography technique: column PRPI (5 μm; Hamilton) 150 × 3.9 mm, mobile phase: borate buffer 0.05 M pH 11.5/acetonitrile (7/3 v/v); flow rate, 0.8 ml/min; detection by fluorescence; excitation 270 nm, emission 418 nm. The limit of detection is 0.01 μg GF120918/g wet tissue.

**Pharmacokinetics of Doxorubicin.** Male mice (C57BL/6 × DBA/2 F1) received 10 mg/kg doxorubicin by i.p. route with or without pretreatment by 10 mg/kg GF120918 i.v. administered 1 h before doxorubicin.

At every point, 10 reference and 10 treated animals were sacrificed. Blood and the following organs were taken: heart; liver; lungs; and kidneys. For each organ doxorubicin was extracted at pH 9 by CHC13 MeOH (4:1) and diethyl ether and assayed by the following high pressure liquid chromatography technique (16): column Novapak C18 (3 μm; Waters) 150 × 3.9 mm; mobile phase, forrniate buffer (4%) pH = 4.0/acetonitrile (7/3 v/v); flow rate, 0.8 ml/min; detection is 0.01 μg doxorubicin/g wet tissue.

**RESULTS**

**Sensitization of Various Cell Lines to MDR Cytotoxics**

The paradigm of MDR inhibitors is the sensitization of MDR cell lines to killing by the MDR cytotoxics. This activity was studied in three different cell lines: CHRC5 a chinese hamster ovary line selected with doxorubicin, OV1/DXR derived from an ovarian carcinoma (12) and MCF7/ADR derived from mammary carcinoma (10).

**Table 1 Sensitization of MDR cell lines by GF120918 and verapamil**

<table>
<thead>
<tr>
<th>MDR inhibitor</th>
<th>CHP(C5)</th>
<th>MCF7/ADR</th>
<th>OV1/DXR</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD50 doxorubicin (μM)</td>
<td>LD50 vinblastine (μM)</td>
<td>LD50 doxorubicin (μM)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>10.2</td>
<td>280</td>
<td>173</td>
</tr>
<tr>
<td>Verapamil, 5 μM</td>
<td>0.18 (56)</td>
<td>4.5 (62)</td>
<td>0.4 (43)</td>
</tr>
<tr>
<td>Verapamil, 1 μM</td>
<td>2.9 (35)</td>
<td>50 (56)</td>
<td>1.4 (12)</td>
</tr>
<tr>
<td>GF120918, 0.5 μM</td>
<td>0.035 (290)</td>
<td>1 (280)</td>
<td>0.26 (67)</td>
</tr>
<tr>
<td>GF120918, 0.1 μM</td>
<td>0.043 (249)</td>
<td>1.28 (280)</td>
<td>0.23 (75)</td>
</tr>
<tr>
<td>GF120918, 0.05 μM</td>
<td>0.074 (190)</td>
<td>1.5 (190)</td>
<td>0.33 (52)</td>
</tr>
<tr>
<td>GF120918, 0.02 μM</td>
<td>0.125 (62)</td>
<td>5.5 (51)</td>
<td>0.37 (47)</td>
</tr>
<tr>
<td>GF120918, 0.01 μM</td>
<td>0.96 (10)</td>
<td>120 (2.3)</td>
<td>3.9 (44)</td>
</tr>
</tbody>
</table>

* *Numbers in parentheses, sensitization factors (LD50 no MDR inhibitor/LD50 MDR inhibitor).*

**Table 2 Doxorubicin sensitization by MDR inhibitors in multidrug-resistant Chinese hamster ovary cells (CHP(C5))**

<table>
<thead>
<tr>
<th>MDR inhibitor</th>
<th>EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiodarone</td>
<td>2.3 ± 0.6 (n=33)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>3</td>
</tr>
<tr>
<td>Quinidine</td>
<td>25</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>2</td>
</tr>
<tr>
<td>GF120918</td>
<td>0.021 ± 0.005 (n=33)</td>
</tr>
</tbody>
</table>

* *Drug level which, combined with 5 μg/ml doxorubicin, results in 50% cell kill of CHP(C5) (72 h exposure); mean ± SD.*

**Table 3 Sensitization to various cytotoxics by GF120918**

<table>
<thead>
<tr>
<th>LD50 (μM)</th>
<th>CHP(C5)</th>
<th>MCF7/ADR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alone</td>
<td>With GF</td>
<td>Alone</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>&gt;800</td>
<td>0.2</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>&gt;800</td>
<td>0.25</td>
</tr>
<tr>
<td>Vincristine</td>
<td>250</td>
<td>18</td>
</tr>
<tr>
<td>Etoposide</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>300</td>
<td>180</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>300</td>
<td>180</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>300</td>
<td>180</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>6.5</td>
<td>0.55</td>
</tr>
<tr>
<td>Vinorelbine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Granulocidin D</td>
<td>40</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Colchicine</td>
<td>30</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>30</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Aclarubicin</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>1.3</td>
<td>0.25</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>78</td>
<td>78</td>
</tr>
<tr>
<td>Fluorouracil</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>650 &gt;650</td>
<td>0.12</td>
</tr>
<tr>
<td>Carmustine</td>
<td>650 &gt;650</td>
<td>0.12</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>400</td>
<td>400</td>
</tr>
</tbody>
</table>

* *ND, not done; NA, not active at maximally tested concentration.*

Indeed, when the sensitizing effect is measured at a fixed 8.6 μM (5 μg/ml) doxorubicin concentration, the median effective dose of GF120918 for sensitization is consistently around 0.02 μM, 100–1000-fold more potent than prototype MDR inhibitors (Table 2). There is a very wide separation between the sensitizing effect and the intrinsic toxicity of GF120918 which had an LD50 of about 27 μM on CHP(C5) cells.

To study the MCF7/ADR cell line, we turned to vinblastine, another MDR cytotoxic, which gives reliable results. The potency of GF120918 and of other MDR inhibitors is consistent with that observed on the two other cell lines (Table 1) and confirms a >100-fold increased potency of GF120918 relative to prototype inhibitors.

We also checked a number of supposedly MDR negative cell lines: the sensitivity of parental MCF7 cells to vincristine (LD50, 1 μM) was unchanged by 0.5 μM GF120918. Similarly, the sensitivity to doxorubicin of the T-cell line Jurkat (LD50, 80 μM) and of the Epstein-Barr virus transformed lymphoblastoid cell line PE117 (LD50, 25 μM) was not affected by GF120918. A 1.6-fold sensitization of HepG2 cells to doxorubicin (from 80 to 50 μM) was observed.

**Cytotoxics Sensitized by GF120918**

In order to determine the spectrum of activity of GF120918, cytotoxicity curves on CHP(C5) and MCF7/ADR for various cytotoxics were constructed in the absence or in the presence of 50 μM GF120918 (Table 3). MDR cytotoxics only are sensitized by GF120918. Sensitization factors vary according to cytotoxics: it is only 2 to 3 for...
Fig. 2. Uptake of daunorubicin by CHRC5 cells. Cells were cultured for 4 h with 0.7 µM [3H]-daunorubicin in the absence (MDR inhibitor, 0) or in the presence of various concentrations of the following MDR inhibitors: GF120918 (○); amiodarone (●); verapamil (■); quinidine (■). Mean ± SD (n = 3).

Uptake and Efflux of Daunorubicin

Uptake of Daunorubicin. The MDR phenotype is characterized by a defect in the accumulation of MDR cytotoxics relieved by MDR inhibitors (1-3). The commercial availability of [3H]daunorubicin allows to set up a straightforward uptake assay to study the effects of MDR inhibitors on drug uptake by MDR cells.

GF120918 enhances up to 4-fold the uptake of [3H]daunorubicin by CHRC5 cells and a near maximal effect is reached at 100 nM GF120918 (Fig. 2). In contrast, well above micromolar concentrations of amiodarone, verapamil, or quinidine are required to enhance to any extent the uptake of daunorubicin.

Efflux of Daunorubicin. Decreased uptake of daunorubicin in MDR cells is attributed to increased efflux mediated by P-glycoprotein, whereas influx is not modified.

Inhibition of P-glycoprotein is achieved reversibly by incubation in glucose-free medium containing azide (see, for example, Ref. 17). Cells are deprived of ATP which is required for the pumping function of P-glycoprotein. This results in enhanced daunorubicin uptake even in the absence of MDR inhibitors.

When cells are placed again in normal medium allowing the regeneration of ATP, very rapid efflux of daunorubicin follows with a half-life of 5 min (Fig. 3). The addition of MDR inhibitors in the medium dramatically slows down the efflux of daunorubicin. Once again GF120918 is active at 20 nM, bringing the half-life of daunorubicin in cells above 4 h. A concentration of verapamil of 10 µM is required for a similar effect (Fig. 3).

Furthermore, if verapamil is present during the uptake phase but omitted from the efflux medium, the efflux is not inhibited. Verapamil has thus a short duration of action in cells probably because it is a substrate of P-glycoprotein (18). In contrast, GF120918 is still active in inhibiting efflux even if it is omitted in the efflux medium. This indicates that GF120918 retained in cells remains in its active form for several hours.

Characterization of P-glycoprotein and Inhibition of Azidopine Labeling by GF120918

The dihydropyridine azidopine has been described as a photoaffinity ligand for calcium channels and P-glycoprotein (19-21). Photoaffinity labeling of cell membranes using radiolabeled azidopine revealed one major band at 170 kD in CHRC5 which is identified as P-glycoprotein by immunoprecipitation and Western blot labeling with P-glycoprotein antibodies such as MDR Ab-1 (Fig. 4A) and JSB-1 (data not shown). Dose-ranging experiments indicate that azidopine labeling is saturable with an apparent Kd of 0.18 µM for CHRC5 P-glycoprotein (data not shown).

As shown in Fig. 4B, the MDR inhibitor GF120918 can compete with azidopine and inhibit radiolabeling: IC50 is observed at 0.16 µM. Similarly, the calcium channel blocker verapamil inhibits azidopine labeling but very high concentration is required (IC50, 45 µM), whereas a cytostatic compound such as vinblastine inhibits the labeling with an IC50 of 4 µM (not shown).

Even if allosteric regulation cannot be totally excluded, these results strongly suggest that GF120918 binds with a high affinity to
Fig. 4. GF120918 inhibits P-glycoprotein labeling by $^{3}$H]azidopine. A, fluorography of a typical experiment where membranes are exposed to various concentrations of GF120918, then to 0.2 $\mu$M $^{3}$H]azidopine and irradiated. B, mean and SD of 4 independent experiments. P-glycoprotein labeling is quantified by scanning the fluorography.

CH$_{10}$P$_{5}$ P-glycoprotein on the same site as the dihydropyridine derivative azidopine.

Pharmacokinetics of GF120918

Blood and tissue levels of GF120918 in mice were assessed after a single i.v. (10 mg/kg) or p.o. (20 mg/kg) administration. Fig. 5 depicts blood levels and heart levels as representative of muscle tissue distribution. The apparent half-life of elimination from blood is estimated to be 2.7 h. Blood levels are generally low but levels in other organs are typically one order of magnitude higher (data not shown for liver, kidney and lungs). This indicates a thorough distribution in most organs except brain where levels are close to that in blood (data not shown).

Blood and tissue levels after p.o. administration indicate a good oral absorption of GF120918.

P388 Leukemia

The P388 leukemia is a classical model for anticancer drugs. The tumor grows i.p. and induces mouse death roughly 10 days after implantation. This model is sensitive to most classical cytotoxics administered by the i.p. route. For example, 1 administration of 2 mg/kg doxorubicin increased mean survival time from 9 to 14 days. Cotreatment with GF120918 10 mg/kg i.v. further increased it to 17 days. This may be due to a low level MDR of this cell line also observed in vitro: the LD$_{50}$ for doxorubicin was decreased from 400 to 70 nm by GF120918.

Several MDR variants have been derived in various laboratories and we have worked on the doxorubicin-resistant variant (P388/Dox) derived by the NCI. The MDR character of this line is confirmed by in vitro experiments showing a 60-fold potentiation by GF120918 of doxorubicin toxicity from 3500 nm to 58 nm.

Effects of GF120918 i.v. in the P388/Dox. As shown in Fig. 6A the tumor is indeed quite insensitive to the maximally tolerated dose of 5 mg/kg doxorubicin. Since the tumor grows i.p. and doxorubicin is administered i.p., there is little rationale in treating i.p. with MDR inhibitors. GF120918 is thus administered i.v., 1 h before doxorubicin injection. This allows time for distribution of the MDR in organs including the peritoneal cavity. In these conditions, GF120918 (10 mg/kg) alone has no effect but, in combination with 5 mg/kg doxorubicin, a dose-related increase in survival time is observed (Fig. 6A). The model proved to be extremely reproducible in a series of consecutive experiments so that the results from these experiments could be pooled yielding accurate and statistically significant results. A dose-dependent sensitization by GF120918 is featured in Fig. 6B: the effect is minimal at 1 mg/kg; half maximal at 2.5 mg/kg; and maximal at 5 mg/kg with a 50% increase in the mean survival time and a 5–10% proportion of 30-day survivors.

It should be pointed out that the above regimen is not intended to maximize the effect of GF120918: a 10 mg/kg bolus at $t = -1$ h yields in mice a $0.14 \mu$g/ml GF120918 blood level at the time of doxorubicin injection (Fig. 5). An infusion regimen would certainly be more relevant to the clinical use of GF120918 and yield better results but is not practical in mice.

Fig. 5. Tissue distribution of GF120918 in mice. A, i.v. administration (10 mg/kg). C, heart; $\bigcirc$, blood. B, p.o. administration (20 mg/kg). C, heart; $\bigcirc$, blood. Mean + SD, $n = 3$. (Fig. 5. Tissue distribution of GF120918 in mice. A, i.v. administration (10 mg/kg). C, heart; $\bigcirc$, blood. B, p.o. administration (20 mg/kg). C, heart; $\bigcirc$, blood. Mean + SD, $n = 3$.
p.o. Treatment. GF120918 is p.o. bioavailable in mice (Fig. 5). An effect in the P388/Dox model is thus expected and is indeed observed in the dose-ranging study described in Fig. 7. Although no direct comparisons are made, GF120918 seems to be 2-3 times less potent by the p.o. than by the i.v. route. Once again, it can be stressed that the regime (GF120918 1 h before doxorubicin) is in no way optimized.

C26 Colon Carcinoma

This mouse cell line has the advantage of being implanted s.c. from cultured cells and having, as many colon carcinomas, spontaneously a MDR character. In vitro sensitization by GF120918 of doxorubicin cytotoxicity is a good measurement of the MDR character: 100 nM GF120918 enhances by about 2-fold the sensitivity to doxorubicin, from 1.1 to 0.5 μM, showing a moderate MDR that may be present in many human tumors.

C26 s.c. growth of the C26 tumor in syngenic mice is affected little by a single dose of doxorubicin (5 mg/kg i.p. at day 1) or by GF120918 alone (10 mg/kg i.p.). In contrast, pretreatment by GF120918 1 h before doxorubicin consistently decreases tumor growth as assessed at days 16 to 19 (Fig. 8). In the absence of GF120918, a 7.5 mg/kg dose of doxorubicin is not more efficacious than 5 mg/kg but toxicity becomes limiting (data not shown). This is an indirect indication that the sensitizing action of GF120918 is likely to be genuine MDR reversal and not pharmacokinetic enhancement of doxorubicin levels.

Lack of Pharmacokinetic Interaction

We addressed in a more direct fashion the issue of modified distribution and/or elimination of doxorubicin as a possible explanation for part of the sensitizing effect. We verified on B6×D2 mice (the strain used for P388/Dox experiments) the effects of a pretreatment by GF120918 (10 mg/kg i.v.) on doxorubicin distribution and elimination from tissues. Table 4 shows no apparent difference in the distribution of doxorubicin in animals pretreated or not with GF120918. Elimination of doxorubicin is very slow with an estimated half-life well above 24 h. Thus, no effect on elimination will show up in this protocol. Such an effect is, however, extremely unlikely since GF120918 has been fully eliminated from blood and organs after 24 h (data not shown).

DISCUSSION

The first salient feature of GF120918 is its potency as an MDR inhibitor. Activity was consistently obtained with median effective dose around 20 μM on different MDR cell lines of murine, Chinese hamster, and human origin and in a variety of cellular assays: sensitization to MDR cytotoxics; enhancement of uptake; and inhibition of efflux of doxorubicin. In all these assays, GF120918 is about 100–200 times more potent than verapamil and it is also about 2 orders of magnitude more potent than other MDR inhibitors such as amiodarone or cyclosporin (Table 2).

To demonstrate activity in animal models, other MDR inhibitors had often to be administered in multiple dose regimens (see for example Refs. 22 and 23). In many cases (e.g., Ref. 23), the MDR inhibitor was administered i.p. directly to the site where the tumor is implanted. In the case of GF120918, potency in vivo translates in vitro.
**MDR INHIBITOR GF120918**

**Fig. 8.** Effect of GF120918 on the activity of doxorubicin on mice bearing the C26 colon carcinoma. A single treatment was administered on day 1. Individual tumor sizes determined at day 19 are indicated (O), as well as the mean ± SE; P < 0.001 (doxorubicin + GF120918 versus doxorubicin alone).

**Table 4. Lack of effect of GF120918 on the tissue distribution and elimination of doxorubicin in mice**

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>Heart</th>
<th>Liver</th>
<th>Lung</th>
<th>Kidney</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>No pretreatment</td>
<td>0.93 ± 0.18</td>
<td>0.09 ± 0.01</td>
<td>0.52 ± 0.05</td>
<td>0.52 ± 0.06</td>
<td>0.65 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>GF120918X</td>
<td>1.08 ± 0.16</td>
<td>0.11 ± 0.01</td>
<td>0.76 ± 0.09</td>
<td>0.59 ± 0.09</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>1 h</td>
<td>No pretreatment</td>
<td>1.03 ± 0.19</td>
<td>0.08 ± 0.01</td>
<td>0.48 ± 0.04</td>
<td>0.46 ± 0.04</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>GF120918X</td>
<td>0.99 ± 0.16</td>
<td>0.09 ± 0.01</td>
<td>0.63 ± 0.05</td>
<td>0.58 ± 0.06</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>4 h</td>
<td>No pretreatment</td>
<td>0.80 ± 0.08</td>
<td>0.07 ± 0.00</td>
<td>0.58 ± 0.04</td>
<td>0.38 ± 0.02</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>GF120918X</td>
<td>0.98 ± 0.13</td>
<td>0.10 ± 0.01</td>
<td>0.84 ± 0.07</td>
<td>0.54 ± 0.07</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>24 h</td>
<td>No pretreatment</td>
<td>0.69 ± 0.05</td>
<td>0.07 ± 0.01</td>
<td>0.50 ± 0.04</td>
<td>0.42 ± 0.03</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>GF120918X</td>
<td>0.91 ± 0.09</td>
<td>0.10 ± 0.01</td>
<td>0.61 ± 0.06</td>
<td>0.44 ± 0.04</td>
<td>0.17 ± 0.03</td>
</tr>
</tbody>
</table>

* GF120918 (10 mg/kg; i.v.) is administered 1 h before doxorubicin (10 mg/kg; i.p.). Results are expressed as µg doxorubicin/g wet tissue; mean ± SE (n = 10).

**vivo:** a single dose of MDR inhibitor i.v. or p.o. enhances the antitumor effects of doxorubicin. This allows dose-ranging studies to be performed. We have thus devised a very simple model that should allow direct comparison of different classes of MDR inhibitors. The intrinsic toxicity of verapamil (e.g., at 20 mg/kg i.v.) precluded its testing at potentially active levels in this system.

Beyond potency, specificity of GF120918 as a classical MDR inhibitor can also be stressed. Cross-linking studies demonstrated its binding to P-glycoprotein and the spectrum of cellular activities described above point to P-glycoprotein inhibition as the most likely mechanism of action. However, in contrast to verapamil, GF120918 is probably in itself a poor substrate of P-glycoprotein so that it is not pumped rapidly outside cells and has thus an extended duration of action after washing off the extracellular medium.

GF120918 was obtained through a chemical program starting from prototype MDR inhibitors such as amiodarone and verapamil. Chemical modification which increased MDR inhibition was retained but it is important to show that other nonselective effects are not also retained during this improvement process. GF120918 is not by itself a cytotoxic, with nonspecific toxic effects observed at concentrations 3 log above specific MDR inhibition. This is in contrast to niguldipine which has both direct cytotoxic and MDR inhibition activities (24).

Another evidence of specificity is the lack of sensitization of non-MDR drugs and of MDR-negative cell lines such as wild type MCF7 and lymphocytic cell lines. In contrast, cyclosporin derivatives are also sensitizers of non-MDR cells, presumably by nonspecific membrane perturbation (25). When sensitization by GF120918 occurs, e.g., in the case of HepG2 hepatoma or wild type P388, it may be due to the slight MDR character of the cell line. Indeed, sensitization by GF120918 might become a sensitive functional assay for the MDR character of cell lines.

Importantly, Ca²⁺ antagonist activity is no longer present: no relaxation of rat aorta was observed up to 10⁻⁵ M GF120918 (data not shown).

In vivo, potentiation of antitumor drugs can be expected not only through genuine MDR inhibition but also indirectly through pharmacokinetic interactions increasing the tissue level of cytotoxics. Although the sensitizing effect of verapamil (26) and cyclosporin A and SDZ PSC 833 in animal models can at least in part be attributed to pharmacokinetic interactions (27), such as explanation is ruled out in the case of GF120918 showing that the observed effects witness genuine in vivo MDR inhibition.

In conclusion, the potency and specificity of GF120918 make this drug a suitable candidate for testing the clinical potential of MDR inhibition without interference from other pharmacological activities.

**ACKNOWLEDGMENTS**

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In Vitro and in Vivo Reversal of Multidrug Resistance by GF120918, an Acridonecarboxamide Derivative

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