Abstract Okadaic acid as well as other, structurally different, inhibitors of serine/threonine phosphatases 1 and 2A induce apoptosis in pituitary GH3 cells. Incubation with stepwise raised concentrations of okadaic acid resulted in the isolation of cells that were increasingly less sensitive to the cytotoxic effect of this agent. After about 18 months cells were selected that survived at 300 nM okadaic acid, which is about 30 times the initially lethal concentration. This study revealed that a major pharmacokinetic mechanism underlying cell survival was the development of a P-glycoprotein-mediated multidrug resistance (MDR) phenotype. The increase in mRNA levels of the mdr1b P-glycoprotein isoform correlated with the extent of drug resistance. Functional assays revealed that increasing drug resistance was paralleled by a decreased accumulation of rhodamine 123, a fluorescent dye which is a substrate of mdr1-mediated efflux activity. Resistance could be abolished by structurally different chemosensitizers of P-glycoprotein function like verapamil and reserpine but not by the leukotriene receptor antagonist MK571 which is a modulator of the multidrug resistance-associated protein (MRP). Okadaic acid resistance included cross-resistance to other cytotoxic agents that are substrates of mdr1-type P-glycoproteins, like doxorubicin and actinomycin D, but not to non-substrates of mdr1, e.g. cytosine arabinoside. Thus, functional as well as biochemical features support the conclusion that okadaic acid is a substrate of the mdr1-mediated efflux activity in rat pituitary GH3 cells. Maintenance of resistance after withdrawal of okadaic acid as well as metaphase spreads of 100 nM okadaic acid-resistant cells suggested a stable MDR genotype without indications for the occurrence of extrachromosomal amplifications, e.g. double minute chromosomes.

Key words GH3 cells · Okadaic acid · Apoptosis · Drug resistance · Multidrug resistance · Resistance modulation

Introduction

Multidrug resistance (MDR) describes the cellular resistance to unrelated cytotoxic drugs. This phenomenon can be due to different mechanisms including extrusion of the drugs by transporter proteins (Endicott and Ling 1989; Cole et al. 1992), increased detoxification of the drugs (Morrow and Cowan 1990), alteration of targets (Danks et al. 1988) and of apoptotic pathways (Reed 1995). A major mechanism of MDR in tumor cells is the expression of 170-kDa transmembrane transporters, the mdr1-type P-glycoproteins (Endicott and Ling 1989; Silverman et al. 1991). Cells expressing these efflux pumps extrude a variety of cytotoxic drugs including vinca alkaloids, anthracyclines and actinomycin D.

Expression of mdr1-type P-glycoproteins in tumor cell lines is often due to an amplification of the mdr1 genes, which can either be chromosomal or extrachromosomal. The chromosomal amplification has been shown to mediate a stable overexpression of the P-glycoprotein (Riordan et al. 1985) while the extrachromosomal amplifications, e.g. as double minute chromosomes (Schoenlein et al. 1992), are unstable and lost within a few cell division cycles in the absence of the selecting drug.

The polyether fatty acid okadaic acid (OA) produced by marine dinoflagellates is a potent inhibitor of the serine/threonine phosphatases 1 and 2A. OA treatment of rat pituitary GH3 cells (Tashjian et al. 1968) concentration- and time-dependently results in apoptotic cell death (Bøe et al. 1991; Tergau et al. 1997). Concentrations exceeding 25 nM OA induce apoptosis within 24 h while concentrations between 10 nM and 25 nM are cytotoxic after 3–5 days of incubation. We previously reported the selection of GH3 cells resistant to 30 nM OA, which is about three times the initially lethal concentration (Ritz et al. 1997). The present study focuses on the pharmacokinetic mecha-
nism leading to genetically stable resistance at up to 300 nM OA, 30 times the initially lethal concentration.

**Materials and methods**

**Materials.** Reagents for cell culture were purchased from Sigma (Deisenhofen, Germany), CPro (Neustadt/W., Germany), Gibco Life Technologies (Eggenstein, Germany) and BioWhittaker (Heidelberg, Germany). Okadaic acid was obtained from RBI (Sigma, Deisenhofen, Germany). MTT (thiazolyl blue), rhodamine 123, reserpine, verapamil, actinomycin D and cytosine arabinoside were purchased from Sigma. The leukotriene receptor antagonist MK571 (Gekeler et al. 1995) was purchased from Biomol (Hamburg, Germany).

**Cell culture.** GH3 rat pituitary cells were maintained in monolayer culture in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 8% fetal calf serum at 37°C in 5% CO2. Resistant cells were isolated by increasing the OA concentration in the culture medium stepwise starting at 7.5 nM.

**Western blot analysis.** For the detection of P-glycoproteins plasma membrane fractions from 5 × 10^6 cells were prepared by differential ultracentrifugation (Simpson et al. 1983). Fifteen micrograms of protein per lane were loaded on 7.5% polyacrylamide gels and were subsequently blotted onto PVDF membranes (Millipore, Eschborn, Germany). P-glycoproteins were detected by utilizing the primary polyclonal antibody PC03 (Oncogene Research Products, Cambridge, Mass., USA). Peroxidase-conjugated anti-rabbit IgG antibody was used as the second antibody. Blots were developed using the ECL system (Amersham, Braunschweig, Germany) following the manufacturer’s instructions.

**Viability assay.** Cells at 2 × 10^4 cells/well were seeded into a 96-well microtiter plate and incubated for 24 h with various drugs in serum-free insulin-transferrin-selenite DMEM followed by the addition of 10 μl MTT solution (5 mg/ml) and incubation for 60 min. After addition of 100 μ1 20% sodium dodecyl sulfate (SDS) in 0.02 N HCl, cells were lysed for 24 h and MTT conversion measured at 550 nm and 650 nm using a Molecular Devices UV max microplate reader.

**Rhodamine 123 accumulation assay (Hirsch-Ernst et al. 1998).** Cells (4.5 × 10^6) were plated on three dishes (Ø 6 cm) in supplemented DMEM. After 3 days cells were incubated for 3 h with 2.5 μg/ml rhodamine 123 in supplemented DMEM and were subsequently washed five times with phosphate-buffered saline (PBS). Cells were lysed for 10 min with 2.5 ml 1-butanol per dish at room temperature. Lysed cells were centrifuged at 1,600 g for 5 min. One milliliter of the supernatant was measured fluorimetrically (excitation at 488 nm, emission at 531 nm). The protein content was determined using the Lowry method (Lowry et al. 1951).

**Northern blot analysis.** Total RNA of 5 × 10^6 cells was isolated using the Quick Prep Total RNA Extraction Kit from Pharmacia (Freiburg, Germany). Eight micrograms of total RNA/lane were run on a 1% agarose gel and blotted onto polyamide membranes. The cross-linked membrane was probed with an mdr1b-specific oligonucleotide (Hirsch-Ernst et al. 1998) and reprobed with a β-actin-specific oligonucleotide (Oncogene Research Products, Cambridge, Mass., USA).

**Preparation of metaphase spreads (Howell et al. 1984).** Cells were treated with 10 μg/ml colchicine for 3 h, trypsinized and incubated in 75 mM KCl for 30 min. Cells were then fixed by incubation on ice in methanol/glacial acetic acid (3:1) for 3 × 30 min. Cells were spread on ice-cold microscope slides and dried for 1 h at 60°C. After incubating the slides in 0.04% trypsin for 30 s, Giemsa staining was performed for 1 h in 50 ml of 10 mM KH2PO4/K2HPO4, pH 6.8, containing 1.5 ml concentrated Giemsa solution (Merck, Darmstadt, Germany).

**Results**

Following a 24-h treatment with OA, rat pituitary GH3 cells die by apoptosis at concentrations exceeding 25 nM (Tergau et al. 1997). Apoptotic cell death was shown to be dependent on the agent’s potency to inhibit protein serine/threonine phosphatases (PP), most likely PP1 and PP2A. Incubation for longer periods than 24 h was lethal at much lower concentrations. As shown in Fig. 1A after 7 days, viability was decreased to less than 10% at OA concentrations around 7.5 nM with virtually no survivors at 10 nM. In order to isolate resistant cell populations, we incubated cells with 7.5 nM OA and subsequently raised the concentration stepwise (Fig. 1B). After about 3–4 months two populations (S1 and S2) were isolated that survived at 30 nM OA, a concentration which was initially lethal to 50% of cells within 48 h. In immunoblots both lines revealed a strongly increased P-glycoprotein expression, with S1 cells displaying the stronger signal (Ritz et al. 1997). Functional investigations revealed that the cell line S2 displayed a comparable or even higher resistance to structurally different phosphatase inhibitors and in addi-