Enhanced expression of human ABC-transporter tap is associated with cellular resistance to mitoxantrone

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Abstract Multidrug resistance (MDR) phenotypes have been associated with the overexpression of various members of the superfamily of ATP binding cassette (ABC) transporters. Here we demonstrate that a member of the ABC-transporter family, the heterodimer 'transporter associated with antigen processing' (TAP), physiologically involved in major histocompatibility complex class I-restricted antigen presentation, is significantly overexpressed in the human gastric carcinoma cell line EPG85-257RNOV exhibiting a mitoxantrone-resistant phenotype. This tumor cell line shows an atypical MDR phenotype in the absence of 'P-glycoprotein' or 'MDR-associated protein' overexpression but with an enforced 'breast cancer resistance protein' expression level. Transfection of both TAP subunits encoding cDNA molecules, TAP1 and TAP2, into the drug-sensitive parental gastric carcinoma cell line EPG85-257P conferred a 3.3-fold resistance to mitoxantrone but not to alternative anti-neoplastic agents. Furthermore, cell clones transfected with both, but not singularly expressed TAP1 or TAP2, reduced cellular mitoxantrone accumulation. Taken together, the data suggest that the heterodimeric TAP complex possesses characteristics of a xenobiotic transporter and that the TAP dimer contributes to the atypical MDR phenotype of human cancer cells. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Mitoxantrone; Atypical multidrug resistance; ATP binding cassette transporter; Cancer chemotherapy

1. Introduction

The multidrug resistance (MDR) phenotype typically occurs after exposure to chemotherapeutic agents. The MDR phenomenon can result from the overexpression of members of the ATP binding cassette (ABC) superfamily of transmembrane transporters that mediate the ATP-driven unidirectional transport of a broad range of compounds across biological membranes. Evidence is emerging that the molecular basis of acquired MDR is multifactorial. Historically, a MDR phenotype that is accompanied by enhanced activity of the ABC-transporter 'P-glycoprotein' (P-gp) [1] is designated as classical MDR, while MDR phenotypes in the absence of P-gp are named atypical MDR or non-P-gp-mediated MDR. Various mechanisms conferring atypical MDR have been described, including overexpression of alternative ABC-transporters, e.g. members of the MDR protein' (MRP) family (overview in [2]) or breast cancer resistance protein (BCRP) (overview in [3]).

To address the problem of atypical MDR, we previously established a cell culture model in vitro. The human gastric carcinoma cell line EPG85-257P exhibiting a high sensitivity against various antitumor drugs was chosen to select the atypical MDR variant EPG85-257RNOV [4] and the classical MDR variant EPG85-257RDB [5] by treatment with stepwise increasing concentrations of mitoxantrone or daunorubicin. Characterization of the atypical MDR phenotype in the gastric carcinoma cells revealed a vesicular compartmentalization of mitoxantrone in the absence of P-gp production [3,4] or distinct MRP1 overexpression [6]. Furthermore, it could be demonstrated that the BCRP encoding mRNA is much more pronounced in the atypical MDR cell line EPG85-257RNOV when compared to the parental, non-resistant cells or the classical MDR derivative [3,7]. However, the current hypothesis of acquired atypical MDR is based on the concept that this phenotype consists of various molecular mechanisms that are simultaneously active. Hence, mechanisms, as yet unknown, may contribute to the atypical MDR phenotype exhibited by the gastric carcinoma cell line EPG85-257RNOV.

The 'transporter associated with antigen processing' (TAP) represents an additional member of the ABC-transporter superfamily (overview in [8]). TAP, a heterodimer formed by TAP1 and TAP2 subunits, physiologically plays a major role in major histocompatibility complex (MHC) class I-restricted antigen presentation by mediating peptide translocation over the endoplasmic reticulum (ER) membrane [9]. TAP1 and TAP2 are homologous polypeptides each possessing a hydrophobic N-terminal domain and a C-terminal ABC domain. Both monomers are required for peptide binding and translocation, preferentially peptides of 8–15 amino acid residues. It has been reported that overexpression of TAP could be detected in MDR cell lines by using a TAP1-specific antiserum [10]. Moreover, the study demonstrated that expression of rat cDNAs encoding TAP1 and TAP2 subunits in the

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TAP-deficient lymphoblastoid cell line T2 could lead to a slightly elevated tolerance to etoposide. In this study, we investigated the role of the human TAP in an acquired atypical MDR phenotype that was induced by mitoxantrone exposure of human gastric carcinoma cells in vitro and postulate that the TAP heterodimer exhibits characteristics of a xenobiotic transporter and contributes to the development of an atypical MDR phenotype in human cancer cells.

2. Materials and methods

2.1. Cell culture

The establishment and characterization of the human gastric carcinoma cell line EPG85-257P and their corresponding atypical MDR subline EPG85-257RNOV and their classical MDR variant EPG85-257RDB have been described previously [4,5]. These tumor cells were grown in Leibovitz L 15 medium (Bio Whittaker, Walkersville, MD, USA) supplemented by 10% fetal calf serum (Gibco BRL, Grand Island, NY, USA), 1 mM L-glutamine, 6.25 mg/l fetuin, 80 IE/l insulin, 2.5 mg/l transferrin, 1 g/l glucose, 1.1 g/l $NaHCO_3$, 1% minimal essential vitamins, and 20 000 kIE/l trasylol in a humidified atmosphere of 5% CO_2 at 37°C. Additionally, the classical MDR line EPG85-257RDB was grown by adding 2.5 μg/ml daunorubicin and the atypical MDR variant EPG85-257RNOV was grown at mitoxantrone concentrations of 0.2 μg/ml. Transfected TAP1 cDNA expression vector containing cell clones were cultivated by adding 400 μg/ml G418 (Invitrogen, San Diego, CA, USA), transfected TAP2 cDNA expressing clones were cultivated in the presence of 400 μg/ml zeocin (Invitrogen), and TAP1 and TAP2 cotransfected clones were grown in the presence of both antibiotics.

2.2. Drugs

The following cytotoxic and antibiotic agents were used: mitoxantrone (Lederle, Wolfratshausen, Germany); daunorubicin and doxorubicin (Farmitalia Carlo Erba, Freiburg, Germany); cisplatin, etoposide and paclitaxel (Bristol-Myers, Munich, Germany); vincristine (Lilly, Giessen, Germany); G418 and zeocin (Invitrogen, San Diego, CA, USA).

2.3. Northern blot analysis

Northern blot analyses were performed as described previously [11]. Briefly, total RNA was prepared using RNAzol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions. 20 μg of total cellular RNA were fractionated on 1% agarose–formaldehyde gels and transferred onto a Hybond-N membrane (Amersham, Aylesbury, UK). Blots were hybridized with a 2.8 kb TAP1 or a 2.5 kb TAP2 encoding cDNA probe labeled with [^{32}P]dCTP by random primed labeling (Amersham). Blots were incubated with 25 ng labeled cDNA probe in 6×SSPE, 10% dextran sulfate, 7% SDS and 0.5% Blotto at 65°C, for 16 h. Finally, the membrane was washed under high stringency conditions (0.1×SSC, 1% SDS at 60°C). As control for the fact that equivalent amounts of RNA were analyzed, the membranes were rehybridized with a 1.2 kb 'phosphoglycerate kinase' (PGK) cDNA probe.

2.4. Western blot analyses

For detection of TAP1 and TAP2, proteins were isolated as described previously [11]. In brief, cellular extracts were prepared by lysing 10^8 exponentially growing PBS-washed cells in Ripa lysis buffer (50 mM Tris–HCl (pH 7.4), 5 mM EGTA, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% (w/v) SDS) supplemented with 1% (w/v) trasylol and Complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) for 30 min at 4°C. The cell lysate was centrifuged at 20 000×*g* for 10 min at 4°C. Supernatant was merged 1:4 with sample buffer (120 mM Tris–HCl (pH 6.8), 300 mM DTT, 4% SDS, 20% glycerol, 0.3% bromophenol blue), heated for 10 min at 98°C, centrifuged at 20 000×*g* for 5 min at 4°C and stored at −80°C. Before SDS–PAGE protein extracts were quantitated using amido black staining [12]. Samples of 20 μg of proteins each were loaded onto a 10% SDS–PA gel. Separated proteins were transferred to a 0.2 μm cellulose nitrate membrane (Schleicher and Schuell, Dassel, Germany). To avoid unspecific binding, the filters were incubated in 5% non-fat dry milk, 0.1% Tween 20 in TBS overnight. Subsequently, filters were incubated with mouse monoclonal antibody 148.3 directed against TAP1 [13], or mouse monoclonal antibody 429.3 directed against TAP2 [14] 1:20 diluted in the same solution for 2 h and, afterwards, with peroxidase-conjugated rabbit anti-mouse IgG (1:5000) (Jackson Immunoresearch Laboratories, West Grove, PA, USA). The protein–antibody complexes were visualized by chemoluminescence (ECL system, Amersham) according to the manufacturer's protocol. Immunoblot reactions were carried out using at least three independent protein extracts.

2.5. Preparation of TAP containing crude membranes and peptide binding assay

Cells were thawn on ice in 10 mM Tris pH 8.0 supplemented with protease inhibitors (50 μg/ml AEBSF, 1 μg/ml aprotinin, 150 μg/ml benzamidine, 10 μg/ml leupeptin, 5 μg/ml pepstatin) and 1 mM DTT and homogenized with a glass homogenizer. Lysed cells were centrifuged at 1000×*g* for 10 min. For collection of crude membranes supernatant was centrifuged at 200 000×*g* for 30 min and pellets were resuspended in PBS. Protein concentrations of the crude membranes were determined by micro bicinchoninic acid protein assay (Pierce). Aliquots were frozen in liquid nitrogen and stored at −80°C. Peptide binding to the heteromeric TAP molecule was assayed as reported previously [15] with the difference that the peptide was labeled with ^{125}I instead of fluorescein.

2.6. Transfection and enforced expression of TAP1 and TAP2 in EPG85-257P cells

For construction of TAP1 and TAP2 eukaryotic expression vectors the 2.8 kb TAP1 encoding cDNA (both TAP monomer encoding cDNA clones were kindly provided by J. Trowsdale, Immunology Division, Department of Pathology, Cambridge, UK) was cloned into the *Xho*I site of the mammalian expression vector pcDNA3 (Invitrogen). The TAP1 cDNA does not contain the frameshift of the originally published clone [16]. For the expression of TAP2, a 2.5 kb cDNA *Xho*I fragment which represents a RING11A allele [17] was cloned into the pcDNA3.1Zeo(+) (Invitrogen) expression vector. Following subcloning of the constructs, a DNA sequence analysis was performed to confirm that the inserts of the selected clones were in a sense orientation to the cytomegalovirus (CMV) promoters of the vectors. Both TAP subunit encoding expression constructs were simultaneously transfected into the parental, drug-sensitive human gastric carcinoma cell line EPG85-257P using liposome transfer (DMRIE, Qiagen, Hilden, Germany). Stably transfected cell clones were obtained by using G418 and zeocin as selecting agents. As controls, EPG85-257P cells were also transfected with unique TAP1 or TAP2 constructs and empty expressing vectors. Subclones were tested for expression of TAP1 and TAP2 encoding mRNAs by RT-PCR and Northern blot analysis.

2.7. Cytotoxicity assay for cell survival

Chemoresistance was tested using a proliferation assay basing on sulforhodamine B (SRB), a protein binding reagent [18]. Cells were distributed into 96 well plates at a concentration of 200 cells/well. For adhesion and resumption the cells in the logarithmic growth phase were incubated for 48 h prior to drug application. After 5 days of drug treatment, the assay was terminated by removal of the media and the addition of chilled 10% trichloroacetic acid. After a 1 h incubation at 4°C wells were washed five times with tap water and cell-associated protein was stained by adding 0.4% SRB in 1% acetic acid for 10 min at room temperature. Absorbance was measured at 540 nm after drying and re-solubilization in 20 mM Tris–HCl (pH 10). For determination of IC_{50}-values of antitumor agents, cells were incubated with increasing concentrations of drugs, whereby the absorbance difference of control cells without drug was set to be 100%. Graphs of cell survival against dose of anti-neoplastic agent were plotted, and IC_{50}-values were calculated from multiple, at least three independent experiments for each cell clone.

2.8. Mitoxantrone accumulation assay

Cellular mitoxantrone accumulation was determined according to a procedure described by others [19]. Briefly, 4×10^5 cells were seeded out in 6 well plates 24 h prior to the experiment. Incubation with mitoxantrone (0, 1, 5 and 10 μg/ml) was performed for 60 min at 37°C and 70–80% cellular confluence. Cells were washed with ice-cold PBS, trypsinized and resuspended in 4°C medium at a concentration of 4×10^5 cells/ml. Intracellular fluorescence of mitoxantrone

was determined by flow cytometry (Becton-Dickinson, San Jose, CA, USA). The cells were excited at 480 nm and emission was measured at 630 nm. A minimum of 10^4 cells was analyzed for each sample. All assays were performed at least in three independent experiments, each time with triplicate wells.

3. Results

3.1. Expression of TAP1 and TAP2 encoding mRNAs in MDR gastric carcinoma cell lines

In order to verify the hypothesis that the heterodimeric ABC-transporter TAP contributes to the MDR phenotypes exhibited by different MDR human gastric carcinoma cell lines, mRNA expression analyses were performed. As shown in Fig. 1, Northern blot analysis revealed that the expression levels of both TAP monomer encoding mRNAs, TAP1 and TAP2, are much more pronounced in the atypical MDR gastric carcinoma cell line EPG85-257RNOV when compared to the drug-sensitive, parental EPG85-257P cells or the classical MDR subline EPG85-257RDB. Only traces of TAP1 and TAP2 encoding mRNAs could be detected in the tumor cell lines EPG85-257P and EPG85-257RDB. This analysis supported the idea that TAP may be involved in the atypical MDR phenotype, but not in the classical MDR phenomenon.

3.2. Overexpression of TAP1 and TAP2 in transfected gastric carcinoma cells

Transfection experiments using TAP1 and TAP2 cDNA containing expression vector constructs were performed to demonstrate that a drug-resistant phenotype could be transferred to former drug-sensitive cells by the transmembrane transporter TAP. After selection with antibiotics, several stable TAP1 and TAP2 transfectant clones and TAP1/TAP2 cotransfectant clones were obtained. These transfectants demonstrated no gross morphological changes in comparison to the original cell line EPG85-257P (data not shown). Starting from these clones, in each case 10 antibiotics-resistant TAP1/TAP2 cotransfected and simply TAP1 or TAP2 subunit cDNA-transfected clones and control clones containing an empty expression vector were analyzed for mitoxantrone resistance using a cell proliferation assay. Two of 10 TAP1/TAP2 cotransfected clones showed an increased tolerance to mitoxantrone exposure when compared to the non-resistant cell line EPG85-257P. None of the control clones, transfected with TAP1 cDNA, or TAP2 cDNA alone, or an empty expression vector showed any modulation in mitoxantrone resistance. In each case one clone was chosen to examine TAP expression in detail. The CMV promoter-driven overexpression of TAP1 and TAP2 encoding mRNAs in the transfected cell clones

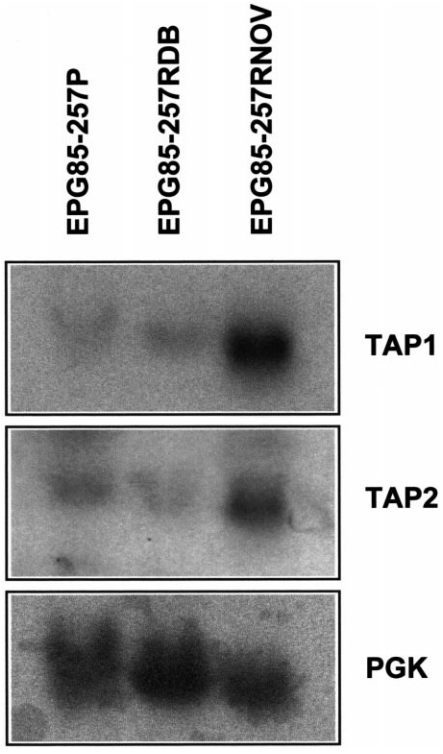

Fig. 1. Northern blot analysis of TAP1 and TAP2 mRNA expression in human gastric carcinoma cells using a 2.8 kb TAP1-specific cDNA probe and a 2.5 kb TAP2-specific cDNA probe. As control, the blots were probed with a 1.8 kb cDNA probe of PGK. The atypical MDR gastric carcinoma cells line EPG85-257RNOV exhibits a distinct overexpression of both TAP subunit encoding mRNAs when compared to the drug-sensitive cell line EPG85-257P and the classical MDR variant EPG85-257RDB.

derived from the human gastric carcinoma cell line EPG85-257P and controls was confirmed by Northern blot analysis on the mRNA level (Fig. 2A) and Western blot analysis on the protein level (Fig. 2B). These analyses demonstrated that an elevated expression level of the TAP1 encoding mRNA could be observed in the TAP1 cDNA transfected cell line EPG85-257P/TAP1 and in the double-transfected clone EPG85-257P/TAP1/2. As expected, an enforced mRNA expression level of TAP2 was detected in the TAP2 cDNA transfected line EPG85-257P/TAP2 and the double-transfected cell line EPG85-257P/TAP1/2. The Western blot analysis could demonstrate that the double-transfected cell line EPG85-257P/TAP1/2 showed an enhanced cellular protein content of both TAP subunits, respectively. Furthermore, in the TAP2-transfected clone EPG85-257P/TAP2 and the atyp-

Table 1
Cross-resistance of TAP expressing cells against various anti-neoplastic drugs

Drug	Cell type				
	EP85-257P IC_{50} (RF)	EPG85-257P/ pcDNA3 IC_{50} (RF)	EP85-257P 257P/TAP1 IC_{50} (RF)	EP85-257P 257P/TAP2 IC_{50} (RF)	EP85-257P 257P/TAP1/2 IC_{50} (RF)
Mitoxantrone (ng/ml)	2.81 (1.0)	2.74 (1.0)	3.66 (1.3)	3.11 (1.1)	9.13 (3.3)
Etoposide (ng/ml)	92.75 (1.0)	55.87 (0.6)	70.50 (0.8)	78.60 (0.9)	102.00 (1.1)
Cisplatin (μg/ml)	2.25 (1.0)	2.00 (0.9)	1.85 (0.8)	1.60 (0.7)	1.98 (0.9)
Paclitaxel (ng/ml)	2.45 (1.0)	1.85 (0.8)	1.88 (0.8)	2.14 (0.9)	2.75 (1.1)
Daunorubicin (ng/ml)	4.35 (1.0)	4.05 (0.9)	4.65 (1.1)	4.50 (1.0)	4.50 (1.0)
Vindesine (ng/ml)	0.358 (1.0)	0.370 (1.0)	0.369 (1.0)	0.197 (0.6)	0.430 (1.2)

RF, resistance factor (*x*-fold). RF was calculated by dividing the IC_{50} value of a given drug of a transfected cell line against the IC_{50} value of the non-transfected, parental cell line EPG85-257P.

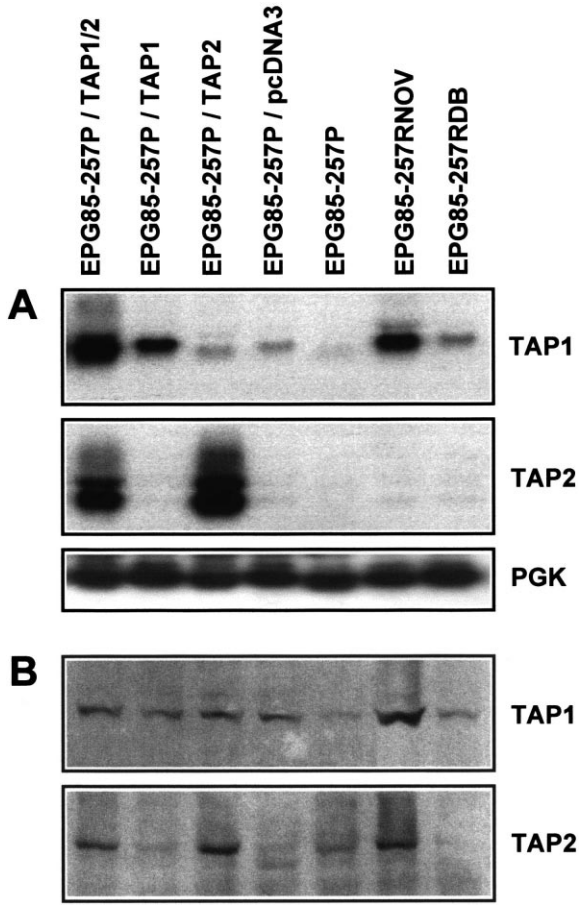

Fig. 2. A: Northern blot analysis of TAP1 and TAP2 mRNA expression in TAP1 and TAP2 cDNA-transfected cell clones derived from the non-resistant, parental gastric carcinoma cell line EPG85-257P. The double-transfected cell line EPG85-257P/TAP1/2 shows expression of both TAP subunit encoding mRNAs while the simply TAP1 or TAP2 transfected clones, EPG85-257P/TAP1 and EPG85-257P/TAP2, only overexpress the corresponding mRNA molecule. Control transfectants, EPG85-257P/pcDNA3, transfected with an empty expression vector, the non-resistant cell line EPG85-257P and the classical MDR cell line EPG85-257RDB show only weak expression of TAP encoding mRNAs. As expected, the atypical MDR line EPG85-257RNOV shows expression of both TAP encoding mRNAs, respectively. In the case of the TAP2 encoding mRNA, the band is not visible due to the short exposure time. Longer exposure time confirmed TAP2 mRNA expression as demonstrated in Fig. 1. As a control, the blots were probed with a PGK cDNA. B: Western blot analysis of TAP1 and TAP2 subunit synthesis in TAP1 and TAP2 cDNA-transfected cell clones derived from the non-resistant, parental gastric carcinoma cell line EPG85-257P. The double-transfected cell line EPG85-257P/TAP1/2 shows a specific protein band of both TAP subunits, respectively. In the TAP2-transfected cell line EPG85-257P/TAP2 and the atypical MDR cell line EPG85-257RNOV, an enforced synthesis of the TAP2 subunit could be detected while the remaining cell lines showed only a constitutive TAP2 polypeptide content. In the TAP1-transfected clone EPG85-257P/TAP1 and in the atypical MDR cell line EPG85-257RNOV, an enhanced cellular content of the TAP1 polypeptide could be detected. As expected the non-resistant, parental cell line EPG85-257P and the classical MDR variant EPG85-257RDB showed only a constitutive cellular TAP1 content while the non-TAP1-transfected controls EPG85-257P/TAP2 and EPG85-257P/pcDNA3 exhibited an increase in cellular TAP1 polypeptide.

ical MDR carcinoma line EPG85-257RNOV, an elevated cellular protein content of the TAP2 polypeptide could be detected while in the remaining cells including TAP1 transfectants and empty vector control transfectants only a constitutive TAP2 content could be observed. Likewise, in the TAP1-transfected clone EPG85-257P/TAP1 and in the atypical MDR cell line EPG85-257RNOV, an increased cellular concentration of the TAP1 subunit could be detected as well as in the non-resistant, parental cell line EPG85-257P and the classical MDR variant EPG85-257RDB a constitutive TAP1 content could be observed. However, in the controls EPG85-257P/TAP2 and EPG85-257P/pcDNA3 an increased cellular TAP1 polypeptide content could be observed even though those clones were not transfected with the TAP1 encoding expression vector construct, indicating that the elevated cellular TAP1 content might be caused by a decreased cellular TAP1 turnover rate, e.g. mediated by stabilization of TAP1 by enhanced complex formation with TAP2.

3.3. Determination of cellular heteromeric TAP

A peptide binding assay was performed to evaluate if the clones overexpressing TAP1 and TAP2 showed an increased cellular content of heteromeric TAP. Fig. 3 demonstrates that a 4.6-fold increased heteromeric TAP content could be detected in the atypical MDR cell line EPG85-257RNOV when compared to the non-resistant, parental cell line EPG85-257P. The classical MDR line EPG85-257RDB showed a slightly 1.9-fold increased heteromeric TAP amount. The TAP1 and TAP2 cotransfected cell line EPG85-257P/TAP1/2 showed a 3.9-fold enhanced heteromeric TAP content while the control clone EPG85-257P/pcDNA3, transfected with an empty expression vector showed no enhanced heteromeric TAP concentration. Likewise, a slightly enhanced heteromeric TAP content could be observed in the simply transfected cell lines EPG85-257P/TAP1 (1.7-fold) and EPG85-257P/TAP2 (1.9-fold).

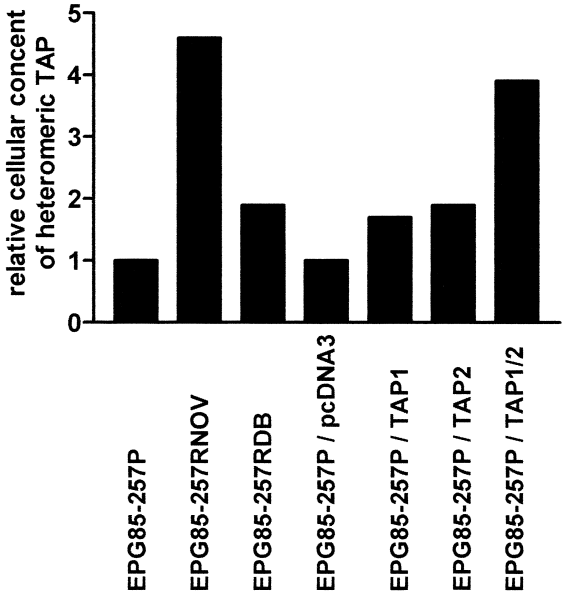

Fig. 3. Relative cellular content of heteromeric ABC-transporter TAP as determined by a peptide binding assay. in the non-resistant, parental cell line EPG85-257P and its atypical MDR derivative EPG85-257RNOV as well as TAP1/TAP2 cotransfected cells and TAP1, TAP2, and empty expression vector transfectants.

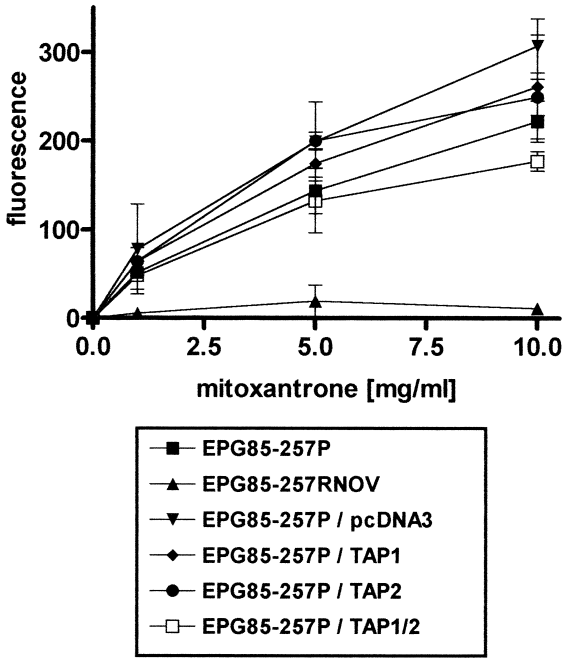

Fig. 4. Accumulation of mitoxantrone as measured by flow cytometry in the non-resistant, parental cell line EPG85-257P and its atypical MDR derivative EPG85-257RNOV as well as TAP1/TAP2 cotransfected cells and TAP1, TAP2, and empty expression vector transfectants. Drug accumulation is shown as a function of increasing concentrations of mitoxantrone after 60 min drug incubation. Displayed values represent the mean, the vertical error bars S.D. of at least three replicate experiments.

3.4. Drug resistance of TAP1 and TAP2 cotransfected cells

The sensitivities of the various transfected sublines to chemotherapeutic agents were tested by a modified SRB-based proliferation assay [18]. As shown in Table 1, it could be demonstrated that the TAP1 and TAP2 cotransfected clone EPG85-257P/TAP1/2 displayed a 3.3-fold resistance to mitoxantrone when compared with the parental, non-resistant gastric carcinoma cell line EPG85-257P. None of the control transfectants, unique TAP1-transfected gastric carcinoma cells EPG85-257P/TAP1, or simply TAP-2 transfected EPG85-257P/TAP2 cells, or clones obtained by transfection with the empty expression vector EPG85-257P/pcDNA3 showed any significant elevated resistance level against mitoxantrone. Likewise, Table 1 demonstrates that no noteworthy cross-resistance against various commonly used anti-neoplastic drugs including etoposide, cisplatin, paclitaxel, daunorubicin or vindesine could be induced in this cell culture model by cotransfection with human TAP1 and TAP2 encoding cDNAs.

3.5. Mitoxantrone accumulation in TAP1 and TAP2 expressing gastric carcinoma cells

As shown in Fig. 4, it could be demonstrated that mitoxantrone-resistant atypical MDR gastric carcinoma cells EPG85-257RNOV exhibited no significant accumulation of mitoxantrone whereas in the drug-sensitive parental cell line EPG85-257P a considerable mitoxantrone-specific fluorescence could be observed. However, the TAP1 and TAP2 cDNA cotransfected gastric carcinoma clone EPG85-257P/TAP1/2 slightly decreased the mitoxantrone-specific fluorescence signal. The unique TAP monomer, TAP1 or TAP2, cDNA transfected cells or the empty expression vector transfected controls showed a mitoxantrone accumulation that was comparable with mitoxantrone accumulation observed in the non-transfected, non-resistant, parental cell line EPG85-257P.

4. Discussion

In this study, we investigated the hypothesis that an increased expression of the ABC-transporter TAP might be involved in the acquired drug resistance phenotype exhibited by the atypical MDR human gastric carcinoma cell line EPG85-257RNOV. Primarily, we could demonstrate that the expression of both TAP subunit encoding mRNAs, TAP1 and TAP2, is much more pronounced in these atypical MDR cells, indicating that the TAP complex indeed contributes to the acquired drug-resistant phenotype exhibited by this gastric carcinoma cell line. In order to demonstrate that TAP could mediate drug resistance to formerly drug-sensitive cancer cells, we cotransfected the parental human gastric carcinoma cell line EPG85-257P with CMV promoter-driven human TAP1- and TAP2-encoding cDNA expression vector constructs. By this approach the TAP1 and TAP2 mRNA and protein expression levels were considerably enhanced in the transfected clones when compared to the non-transfected cells. Applying this experimental strategy it was possible to achieve a cellular heteromeric TAP polypeptide content in the transfected clones that was similar to that TAP level observed in the drug-resistant cell line with acquired atypical MDR. TAP1 and TAP2 overexpressing cells derived from the cell line EPG85-257P showed a 3.3-fold increase in mitoxantrone resistance at the IC_{50} level compared to non-transfected EPG85-257P cells and controls accessed by cell proliferation assays. The fact that several TAP1 and TAP2 cotransfected clones and none of the controls increased their tolerance to mitoxantrone exposure supports the idea that the new phenotype exhibited by the cotransfected clones is indeed caused by TAP overexpression and not by unspecific clonal effects. The data presented here strongly support the conclusion that the ABC-transporter protein family member TAP possesses characteristics of an ATP-dependent xenobiotic transporter that plays a role in the atypical MDR phenotype of human gastric carcinoma cells. The overexpression of both TAP monomers in the atypical MDR cell line EPG85-257RNOV suggests a role for TAP in resistance to anti-neoplastic agents in human cancer cells.

TAP overexpression is, in general, paralleled by an enhanced expression of MHC I molecules. Since no increase in MHC I expression could be observed in the atypical MDR cell line EPG85-257RNOV or in any of the TAP1 or TAP2 transfected or cotransfected clones (data not shown), it seems obvious that the TAP-mediated alteration in mitoxantrone tolerance in these cells is an MHC I independent phenomenon.

The exact molecular events by that the TAP-mediated alterations in mitoxantrone susceptibility are effected are not understood in detail. However, it could be demonstrated that the enhanced mitoxantrone resistance of TAP1 and TAP2 cDNA cotransfected clones is accompanied by a decreased cellular mitoxantone-specific fluorescence indicating that the drug was exported from the cells. Physiologically, the heterodimeric TAP complex is located on intracellular membranes of the ER. TAP translocates short peptides which were prior generated mostly by proteasome-mediated antigenic protein degradation in the cytosol, into the lumen of the

ER. In the ER lumen, the TAP-complexed peptides interact with assembled MHC class I heavy chain-β_2-microglobulin heterodimers, leading to the transfer of the peptides, subsequent TAP dissociation from the complex and export of peptides bound to MHC class I molecules to the Golgi apparatus and ultimately to the cell surface. The human TAP complex shows a preference for peptides with hydrophobic or basic amino acids at the C-terminus. Hence, it is tempting to speculate that the heterodimeric TAP complex may mediate the translocation of hydrophobic antitumor agents, such as mitoxantrone, into the ER lumen. Since cellular vesicles, in general, are derived from the ER, the TAP-mediated drug translocation may be one step in the vesicular compartmentalization of mitoxantrone which was observed in the atypical MDR gastric carcinoma cell line previously [3,4].

In contrast to the observations by Izquierdo et al. [10], who transfected rat TAP complex encoding cDNAs into lymphoblastoid T2 cells and conferred mild resistance to etoposide, vincristine and doxorubicin (2–2.5-fold), our study could not demonstrate that the human TAP dimer mediates resistance to those anticancer agents in gastric carcinoma cells. Since Izquierdo et al. [10] did not analyze the drug sensitivity of their transfected clones against mitoxantrone, it is not clear whether these discrepancies are due to differences in the substrate specificity of human and rat TAP complexes, utilization of cellular models of different entities, or whether the used cDNA expression vector constructs were not adapted for achievement of sufficient cellular amounts of biological active TAP complexes.

The value of a 3.3-fold increase in mitoxantrone resistance in this cellular in vitro model is not dramatic. However, a huge alteration in the drug-resistant phenotype may not to be expected since it was demonstrated that alternative mechanisms such as vesicular compartmentalization [3,4] alterations in DNA topoisomerase IIα expression [6] and enhanced expression of BCRP [3,7] can be associated with the acquired mitoxantrone-resistant phenotype exhibited by EPG85-257RNOV cells. Furthermore, additional candidate factors were identified [20,21] that might play a potential role in the mitoxantrone resistance of these cells. On the other hand, it is important to note that the effect of change in drug tolerance associated with alterations in TAP1 and TAP2 mRNA expression levels has implications and is of clinical significance. In the clinical situation, a 2-fold increase in drug resistance is sufficient to circumvent a successful chemotherapeutic treatment of malignant cells. Thus, for the oncologist it is critical to take into consideration that changes in TAP expression level may be involved in a multimodal mechanism leading to therapy resistance of human cancers.

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