Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and confers resistance to irinotecan \textit{in vitro}

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Abstract

Members of the multidrug resistance – associated protein (MRP) family of transporters are believed to contribute to cytotoxic drug resistance and chemotherapy failure. We observed frequent \textit{MRP4} overexpression in aggressive primary neuroblastoma, a disease for which we have previously shown \textit{MRP1} to be a prognostic indicator. High \textit{MRP4} expression correlated with \textit{MYCN} oncogene amplification and was significantly associated with poor clinical outcome. Although \textit{MRP4} is known to transport some nucleoside analogues, it has not previously been associated with resistance to drugs used to treat solid tumors. We now show that it mediates substantial resistance \textit{in vitro} to the topoisomerase I poison irinotecan/CPT-11 and its active metabolite SN-38. These results suggest that \textit{MRP4} will be a useful prognostic marker for neuroblastoma and that clinical trials of irinotecan as a neuroblastoma treatment should monitor \textit{MRP4} expression. The same may be true for other tumor types expressing high levels of the transporter. [Mol Cancer Ther 2005;4(4):547 – 53]

Introduction

The human multidrug resistance–associated proteins (MRP) are a family of transport proteins that seem to play a critical role in cellular detoxification. Of these, \textit{MRP1}, \textit{MRP2}, and \textit{MRP3} have been shown to confer resistance to a variety of natural products and anticancer drugs (1), including the \textit{Vinca} alkaloids, anthracyclines, epipodophyllotoxins, and camptothecin-class topoisomerase I inhibitors. They also share the ability to transport glutathione and glucuronide conjugates. \textit{MRP4} (ABCC4), the smallest member of this family, has previously been associated with resistance to the nucleoside analogues 6-mercaptopurine and thioguanine as well as antiretroviral compounds (2–4). However, this multidrug transporter has not previously been linked to resistance involving natural product drugs. Of all the MRP-class transporter proteins, \textit{MRP1} has been the most widely studied and best-characterized and available evidence points to a role for \textit{MRP1} in the clinical drug resistance of several malignancies, including neuroblastoma.

Survival rates for neuroblastoma, the most common extracranial solid tumor in young children, remain below 50%. Neuroblastoma frequently displays amplification of the \textit{MYCN} oncogene and this genetic aberration is associated with rapid tumor progression, advanced clinical stage, and poor outcome (5). We have previously shown a close correlation between expression of \textit{MYCN} and the multidrug transporter protein \textit{MRP1} and provided strong evidence of a role for \textit{MRP1} (but not \textit{MDR1} P-glycoprotein) in mediating the drug-resistant phenotype of this aggressive childhood cancer (6). In addition, we have shown that \textit{MYCN} may mediate poor response to treatment by up-regulating expression of the \textit{MRP1} gene (7–9).

We have now examined the expression of other multidrug transporter genes in aggressive primary neuroblastomas and find that \textit{MRP4} (ABCC4) is also often overexpressed. Like \textit{MRP1}, high \textit{MRP4} expression correlates with poor clinical outcome and \textit{MYCN} gene amplification and overexpression. Moreover, we show that \textit{MRP4} confers resistance \textit{in vitro} to the water-soluble derivative of camptothecin, irinotecan/CPT-11, and its active metabolite SN-38. Irinotecan is a potent topoisomerase I poison and has become a drug of interest for the future treatment of a number of cancers, including neuroblastoma.
Materials and Methods

Patient Samples

Samples of 52 primary neuroblastoma tumors from untreated patients, representing all clinical stages, were obtained either from the Neuroblastoma Tumor Bank of the Clinical Oncology Group, USA or from the Sydney Children's Hospital, Sydney, Australia. Of the 52 tumors, 10 were stage I (including five ganglioneuroblastomas), 9 were stage II, 18 were stage III, 12 were stage IV, and 3 were stage IVS. Tumors were grouped into those with favorable (stages I, II, and IVS) or unfavorable (stages III and IV) clinical stage. All samples were taken during the course of the patients' routine management. In each case, the number of copies of the MYCN oncogene per haploid genome had been independently determined by Southern analysis before this study and was found to be amplified in 13 of the tumors (7 stage III and 6 stage IV). All patients were diagnosed between December 1984 and September 1994 and were treated in a stage-specific manner according to protocols previously described (6).

PCR Analysis of Gene Expression

Total cytoplasmic RNA was isolated from frozen neuroblastomas and cDNAs synthesized using random hexanucleotide primers. For conventional PCR, a competitive RNA-PCR assay was used as described previously (6). Gene sequences for each of the multidrug transporters, MRP1 to MRP6 and BCRP, were independently coamplified with the control gene sequence (β2-microglobulin), using the following gene-specific oligonucleotide primers: MRP2 forward primer 5'-ACCAATCCAAGCTTACCTAC- TAG-3', reverse primer 5'-GAAGTGGCACAGATAGTC- GAG-3' (132-bp PCR product); MRP3 forward primer 5'-GCTTTCCTCCGGCCTGTT-3', reverse primer 5'-TTTACGGACACAGGTCTTCTC-3' (93-bp PCR product); MRP4 forward primer 5'-GGAACAAAGACAATCTGG- TGTGCC-3', reverse primer 5'-AATGGTTAGCACGGTG- CATTCGAGGAGTTGTCTTTGTCAA-3' (72-bp PCR product); MRP5 forward primer 5'-CCCTCCGAGAAAGCTCTGTCAT-3' (72-bp PCR product); MRP6 forward primer 5'-CCCCCTCAAAGCAGGCT-3', reverse primer 5'-GCCGGGAAACAAGTTCTTCTG-3' (124-bp PCR product); BCRP forward primer 5'-AAC- CAGTTGAGAAAGAAATATTATGCA-3', reverse primer 5'-CACCCTGTGGTCCAGAGAT-3' (80-bp PCR product). MRP1- and β2-microglobulin–specific sequences have been described previously (6). Following PAGE, the mRNA level of target gene was densitometrically determined using the Hexanucleotide primers. For conventional PCR, a competitive RNA-PCR assay was used as described previously (6). The human embryo kidney cell line HEK293 and transduced sublines HEK293/MRP4 clone 4.63 (3) and HEK293/MRP5 clone 5I (10) were cultured in DMEM containing 10% supplemented calf serum (HyClone, Logan, UT), penicillin and streptomycin (“complete medium”). Cytotoxicity assays were done as described previously (11).

Drug Accumulation Assays

HEK293/MRP4 cells were seeded at 2.5 × 10^6 per well in 6-well plates. Adhesion was improved by precoating plates with 0.1% (w/v) gelatin in PBS for 60 minutes at room temperature and adding 20 mmol/L MgCl_2 to the plating medium. Following attachment overnight, cells were reincubated in 2 mL of fresh complete medium with or without 50 μmol/L dipyridamole for 30 minutes at 37°C. Irinotecan and/or SN-38 were added in 0.5 mL complete medium, to a final concentration of 2 or 1 μmol/L, respectively, and the plates returned to the incubator for 30 minutes. Plates were placed on ice; wells washed once with ice-cold complete medium and once with ice-cold DMEM without serum. Drug was extracted from the adherent cells in situ with 1 mL of 1:1 acetonitrile/methanol by gentle agitation in the dark at room temperature for 30 minutes. The solvent contained 10 mg/mL camptothecin as an internal control for recovery during subsequent steps. The solvent was clarified by centrifugation, lyophilized, resuspended in mobile phase, and analyzed by high-performance liquid chromatography for irinotecan, SN-38, and camptothecin content, as described (12).

Immunoblot Analyses

Cells (4 × 10^7/mL) and tissues (100 mg/mL) were lysed by douncing and sonication in 10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L MgCl_2, 0.5% SDS, and protease inhibitors (Sigma-Aldrich, St. Louis, MO). Lysates (20 μg protein per lane) were analyzed by SDS-PAGE and blotted proteins probed for MRP4 with the monoclonal antibodies 2E10 (similar to 12C4; ref. 3) and M4-10.9 Replicate blots were probed for MRP5 with monoclonal 55I-1 (13) and for α-tubulin with monoclonal YL1/2 (ref. 14; AbCam, Cambridge, United Kingdom). Bound monoclonals were detected with horseradish peroxidase–conjugated goat anti-rat immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by enhanced chemiluminescence (West Pico, PerBio, Rockford, IL).

9 G.L. Scheffer, manuscript in preparation.
Statistical Analysis

MRP4 expression in each individual tumor was categorized as low or high by dichotomizing around the median \( \Delta \Delta C_t \) value obtained from all 52 tumors. Comparisons of outcome between subgroups were done by the log-rank test for univariate comparisons, using two-tailed tests and survival analysis was done according to the method of Kaplan and Meier. Multivariate analysis was done using Cox’s proportional hazards regression model. Survival probabilities and relative hazards are given with 95% confidence intervals (95% CI). Differences between groups of tumor specimens in terms of their gene expression was assessed by Student’s t tests, using two-sided P values. All data were analyzed with StatView 4.1 (Abacus Concepts, Inc., Berkeley, CA) and Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

Results

High MRP4 Expression Is a Prognostic Marker in Neuroblastoma

We have previously shown that MRP1 gene expression is a strong prognostic marker for children with neuroblastoma, whereas MDR1 is not (6). It was therefore of interest to examine the utility of other multidrug transporters in this role. Using semiquantitative reverse transcriptase-PCR, expression of the MRP2, MRP3, MRP4, MRP5, MRP6, and BCRP genes was determined in a panel of 19 primary neuroblastoma samples, specifically selected as a screening tool, which included tumors with clinical and biological features associated with either good or poor clinical outcome. The suitability of using this panel was initially confirmed by examining expression of the MRP1 gene. As anticipated, high levels of MRP1 were significantly associated with MYCN amplified tumors as well as those with a poor outcome (data not shown). However, using this approach for MRP2 to MRP6 and BCRP, only MRP4 emerged as a promising candidate because high levels of expression of this gene, but not of any of the other MRP gene family members, were significantly associated with poor outcome (Fig. 1) and MYCN amplification (data not shown). MRP6 expression was undetectable in the majority of the tumors analyzed. MRP4 expression was found significantly higher in those tumors with MYCN gene amplification (mean PCR ratio = 0.221 ± 0.053) versus those lacking (mean PCR Ratio = 0.063 ± 0.023) amplification (\( P < 0.01 \)). Likewise, MRP4 expression was significantly higher in tumors from those children who subsequently died (mean PCR ratio = 0.206 ± 0.048) by comparison with the tumors from those who survived (mean PCR ratio = 0.060 ± 0.026; \( P < 0.01 \)). In addition, linear regression analysis showed a significant correlation between MRP4 and MRP1 expression in the 19 tumors (\( R = 0.56, P = 0.01 \)).

MRP4 gene expression was then examined in 52 primary untreated neuroblastomas using real-time PCR. In the first instance, survival was analyzed for well-established prognostic variables and, as expected, children with MYCN gene amplification, late clinical stage, and those older than 1 year at diagnosis all had lower rates of event-free survival and overall survival compared with children without amplification, early-stage disease, or ages <1 year, respectively (Table 1). The characteristics of the study population with respect to well-established prognostic indicators and outcome were thus representative of neuroblastoma tumors in general. All the tumors, regardless of clinical stage, were found to express MRP4, although variation in the level of expression was apparent. When patients were dichotomized around the median real-time PCR value, a high level of MRP4 expression was significantly associated with both a reduced event-free survival and overall survival (\( P < 0.001, P < 0.005 \), respectively; Fig. 2). In contrast, when MRP2 expression was examined, there was no significant association between expression of MRP2 and clinical outcome for either survival or event-free survival (\( P > 0.05 \) in each case). The strength of the association between MRP4 gene expression and outcome was confirmed by further dichotomizing around the upper quartile for MRP4 expression. MRP4 expression remained a highly significant predictor of event-free survival and overall survival with a relative hazard of 10 (95% CI, 3–31; \( P < 0.0001 \)) and 47 (95% CI, 6–375; \( P < 0.001 \), respectively.

To determine whether MRP4 gene expression had independent prognostic significance, multivariate analysis was also done. When outcome based on event-free survival was adjusted for the effect of MYCN oncogene amplification, age at diagnosis, and disease stage, only
MRP4 expression remained a significant indicator of poor survival. Thus, high levels of MRP4 expression were independently associated with a significantly higher risk of poor outcome (relative hazard, 9.7; 95% CI, 1.8–53; \( P < 0.01 \)), whereas MYCN (relative hazard, 0.47; 95% CI, 0.1–2.1; \( P = 0.32 \)), age at diagnosis (relative hazard, 0.48; 95% CI, 0.1–1.7; \( P = 0.25 \)), and disease stage (relative hazard, 3.9; 95% CI, 0.8–19; \( P = 0.09 \)) lost all prognostic significance.

MRP4 Confers Resistance to Irinotecan

As camptothecin-type topoisomerase I inhibitors are currently undergoing clinical trials for treatment of neuroblastoma, we assessed the possibility that MRP4 confers resistance to such agents by comparing the sensitivity of HEK293 human embryonic kidney cells with a transfected subclone overexpressing human MRP4 (ref. 3; Fig. 3). ABC transporters expressed ectopically in transfected cell lines are not always efficiently routed to the plasma membrane (15); thus, an unknown (but probably large) fraction of the MRP4 protein detected in the HEK293/MRP4 cells may be cytoplasmic and nonfunctional. Nevertheless, in cytotoxicity assays, the MRP4 transfectants were at least 5-fold more resistant to the topoisomerase inhibitor irinotecan and its active metabolite SN-38 (Table 2). The resistance was similar to that for 6-mercaptopurine, one of the better nucleoside analogue substrates (4, 10). It is interesting to note that although MRP5 confers resistance to many of the same nucleoside analogues as MRP4 (4), this was not the case for irinotecan or SN-38 (Table 2). The MRP4-mediated resistance to irinotecan and SN-38 was relatively specific in thus far as there was no noticeable resistance to two other drugs in the same class, topotecan and camptothecin.

Intracellular accumulation of SN-38 and irinotecan were reduced in the HEK293/MRP4 cells compared with the parent cell line (Fig. 4), as would be expected from MRP4-mediated drug efflux. Intracellular SN-38 was reduced at least 4-fold, whereas accumulation of irinotecan was only modestly reduced, suggesting that it is a less efficient substrate of MRP4 than SN-38. The differences between the HEK293/MRP4 cells and the parent cell line in accumulation of these compounds were significantly diminished or eliminated in the presence of 50 \( \mu \text{mol/L} \) dipyridamole (Fig. 1B), an inhibitor of several MRP-class transporters including MRP4 (4). Similar results were obtained whether the cells were exposed to irinotecan CI, 0.1–1.7; \( P = 0.25 \), and disease stage (relative hazard, 3.9; 95% CI, 0.8–19; \( P = 0.09 \)) lost all prognostic significance.

Table 1. Prognostic variables and outcome

<table>
<thead>
<tr>
<th></th>
<th>( n ) (%)</th>
<th>Event-free survival (%)</th>
<th>( P )</th>
<th>Survival (%)</th>
<th>( P )</th>
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<tbody>
<tr>
<td>MYCN amplification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonamplified</td>
<td>39 (75)</td>
<td>82 (70–94)</td>
<td>0.004</td>
<td>90 (80–99)</td>
<td>0.001</td>
</tr>
<tr>
<td>Amplified</td>
<td>13 (25)</td>
<td>35 (0.1–63)</td>
<td></td>
<td>46 (19–73)</td>
<td></td>
</tr>
<tr>
<td>Disease stage(^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favorable</td>
<td>22 (42)</td>
<td>86 (72–100)</td>
<td>0.04</td>
<td>100 (100–100)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Unfavorable</td>
<td>30 (58)</td>
<td>58 (40–77)</td>
<td></td>
<td>62 (44–80)</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;1</td>
<td>24 (46)</td>
<td>83 (68–98)</td>
<td>0.11</td>
<td>96 (88–100)</td>
<td>0.01</td>
</tr>
<tr>
<td>( \geq 1 )</td>
<td>28 (54)</td>
<td>61 (43–79)</td>
<td></td>
<td>64 (47–82)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Numbers in parens refer to 95% CIs.
\(^b\)Patients were grouped into favorable (stages I, II, and IVS) or unfavorable (stages III and IV) disease stage.

Figure 2. Cumulative event-free survival (A) and survival (B) in 52 neuroblastoma patients according to expression of the MRP4 gene. The level of MRP4 gene expression in each primary tumor was determined by real-time PCR assay (see Materials and Methods) and individual tumors were dichotomized around the median PCR value. Survival curves were analyzed by log-rank test.
and SN-38 together or separately (data not shown), indicating that SN-38 and irinotecan are not competitors for transport by MRP4 at low micromolar concentrations, similar to those obtained in the body during treatment.

Despite the observation that intracellular accumulation of SN-38 was reduced to a greater extent than irinotecan, the HEK293/MRP4 cells were equally resistant to irinotecan and SN-38, as noted above (Table 2). This suggests that the observed resistance to irinotecan and SN-38 reflects efflux of SN-38 in both cases. Irinotecan is at least 100-fold less potent than SN-38; thus, its toxicity in vitro could reflect the presence of low levels of contaminating SN-38 present in clinical formulations of irinotecan and/or low levels of carboxylesterase activity in the HEK293 cells, that would generate SN-38 in situ from the prodrug. Consistent with this latter view, low levels of SN-38 were in fact detected by high-performance liquid chromatography in HEK293 cells treated with irinotecan alone during the accumulation experiments.

Discussion

All primary neuroblastomas examined in this study were found to express MRP4 and its overexpression was significantly associated with MYCN amplification. In addition, MRP4 and MRP1 expressions were found significantly correlated. We have previously provided evidence that MYCN regulates expression of the MRP1 gene (8), and given that drugs routinely used to treat neuroblastoma do not seem to be MRP4 substrates, it is

Table 2. Resistance to irinotecan and SN-38 in MRP4-transduced HEK293 cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Mean IC&lt;sub&gt;50&lt;/sub&gt; ± SD (nmol/L), mean RF ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEK293</td>
<td>HEK293/MRP4</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>8</td>
<td>360 ± 100</td>
</tr>
<tr>
<td>SN-38</td>
<td>8</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Topotecan</td>
<td>7</td>
<td>10.6 ± 2.1</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>8</td>
<td>8.0 ± 1.7</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>8</td>
<td>2,230 ± 770</td>
</tr>
<tr>
<td>Vincristine</td>
<td>4</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

NOTE: Means summarize n independent experiments. Resistance factors were calculated within each experiment. Two-tailed Student’s t tests of H<sub>0</sub>: RF = 1 were performed. P values are shown where statistically significant. Abbreviation: RF, resistance factor.
interesting to speculate that the prognostic significance provided by MRP4 also reflects regulation of its expression by MYCN. We are currently investigating this possibility as several potential MYCN-binding sites (E-boxes) are present in the MRP4 promoter. Alternatively, MRP4 may be transporting some other nondrug substrate important for the maintenance of neuroblastoma malignancy. Given these results, we are currently analyzing the independent prognostic significance of MRP4 and MRP1 gene expression, as well as MYCN gene amplification, in a large cohort of prospectively accrued primary neuroblastoma samples.

The drug resistance phenotype of MRP4 has to date been thought to encompass primarily nucleoside analogues (including antiretroviral agents) and methotrexate (1). In this report, we show that MRP4 is also able to confer significant resistance in vitro to the topoisomerase I poison irinotecan and its active metabolite SN-38. This finding not only broadens the range of drugs known to be substrates of MRP4 but may also have implications for optimal clinical use of irinotecan. This drug is approved for treatment of colon and lung cancers and shows promising activity against a number of other cancers including cervical, ovarian carcinomas, and also neuroblastoma (16). However, the clinical response rates for irinotecan for some cancers have been lower than expected from xenograft models, for reasons that are largely unknown (17). The finding that MRP4 confers resistance to irinotecan in vitro suggests that its expression may be one factor that impinges on the efficacy of Irinotecan in specific tumors. Although caution is required in extrapolating from in vitro studies of drug resistance to tumors in vivo, lower levels of resistance to irinotecan than that observed in the HEK293/MRP4 cells would be clinically significant. Although MRP4 is expressed at low levels in a range of normal tissues including brain, testes, ovary, pancreas, and lung, high-level expression has been observed in both kidney and prostate (1, 18, 19). Hence, it is interesting to note that a recent phase II study of irinotecan in hormone-refractory prostate cancer patients concluded that at the dosage and scheduling used, irinotecan did not have significant activity against this tumor type (20). A similar finding was also reported following a phase II study of irinotecan in patients with advanced renal cell carcinoma (21). Although prostate and renal carcinomas respond poorly to essentially all cytotoxic agents, it will be important to determine whether the limited response to irinotecan can be related to the level of MRP4 in these tumors.

Although MRP4-transfected cells were resistant to irinotecan, a water-soluble derivative of camptothecin, the resistance did not extend to campothecin itself or to the related compound topotecan. This pattern is distinct from resistance mediated by other transporters of camptothecin drugs, including P-glycoprotein (22), BCRP (23), MRP1 (24), and MRP2 (25). In contrast to our results, Schuetz et al. recently reported that MRP4 confers resistance to topotecan using MRP4-transfected Saos-2 cells (26). Because the MRP4 cDNAs in the Saos-2 and HEK293 transfectants have the same origin, the observed discrepancy in resistance to topotecan is somewhat surprising and it will therefore be important to undertake independent comparisons of MRP4-mediated resistance in other models to clarify this issue. One possible explanation for the discrepancy however, relates to the comparatively high levels of other topotecan transporters in the HEK293 cells, including BCRP and MRP1, which may be sufficient to mask MRP4-mediated resistance to this drug. Irrespective, our results suggest that SN-38 is a better MRP4 substrate than topotecan. It will also be of great interest to test whether MRP4 can confer resistance to other experimental camptothecin analogues including 9-aminocamptothecin, 9-nitrocamptothecin, and DX-8951f. Finally, despite the fact that MRP4 and MRP5 exhibit similar profiles of resistance to nucleoside analogue drugs (4), we could find no evidence of MRP5-mediated resistance to any of the camptothecin drugs tested.

In conclusion, the results presented here suggest that irinotecan will be most effective for treating neuroblastomas that lack MYCN amplification due to the lower levels of MRP4 and MRP1 in these tumors, and less effective for neuroblastomas or for other tumors that express significant levels of MRP4. If borne out, this prediction provides an opportunity for improving the therapeutic results already obtained with this promising drug.

Acknowledgments
We thank Pfizer Australia for kindly providing SN-38.

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Molecular Cancer Therapeutics

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Mol Cancer Ther 2005;4:547-553.

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