The H$^+$-Linked Monocarboxylate Transporter (MCT1/SLC16A1): A Potential Therapeutic Target for High-Risk Neuroblastoma

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ABSTRACT

Neuroblastomas produce high amounts of lactic acid and up-regulate the H$^+$-linked monocarboxylate transporter isoform 1 (MCT1/SLC16A1). We found elevated MCT1 mRNA levels in fresh neuroblastoma biopsy samples that correlated positively with risk of fatal disease and amplification of the "proto-oncogenic" transcription factor MYCN. We further investigated MCT as a potential therapeutic target in vitro. The neuroblastoma cell lines evaluated were Sk-N-SH, CHP134, IMR32, and NGP. All lines exhibited decreased intracellular pH at low tumor-like extracellular pH. Lonidamine or exogenous lactate further lowered intracellular pH. Immediate early lowering of intracellular pH with Lonidamine or lactate at extracellular pH 6.5 correlated positively with diminished cell viability within 48 h. These findings indicate that MCT1 is a potential therapeutic target and that neuroblastoma therapy may be enhanced by therapeutic strategies to inhibit or overwhelm MCT. Additional experiments indicated that the mechanism of cell death by lonidamine or exogenous lactate is similar to that obtained using α-cyano-4-OH-cinnamate, a well established MCT inhibitor. Because lactate production is also high in melanoma and many other tumor types, MCT inhibitors may have broad application in cancer treatment. Such treatment would have selectivity by virtue of the acidic milieu surrounding tumors, because MCT is increasingly active as extracellular pH decreases below 7.0 and lactic acid production increases.

Neuroblastomas are childhood malignancies postulated to originate from precursor cells of the postganglionic sympathetic nervous system (Brodeur, 2002). The disease is remarkable for its clinical heterogeneity; spontaneous regression is common in infants diagnosed before the age of 12 months, but relentless malignant progression is common in children diagnosed after their first birthday. This latter category of "high-risk" patients represents 50% of all cases. Moreover, mortality in this subset remains greater than 60% despite aggressive chemoradiotherapy (Matthay et al., 1999; Brodeur, 2002). New treatment approaches are clearly needed.

High lactic acid production is a common feature of many solid-tumor cells (Skoyum et al., 1997; Walenta et al., 1997; Brizel et al., 2001). The transporters responsible for export of lactic acid have been characterized in detail (Halestrap and Meredith, 2004). Furthermore, we have shown that MCT isoform 1 (MCT1) and MCT4 both have elevated activity in human melanoma cells in response to low extracellular pH (Wahl et al., 2002). This suggests that the level and/or affinity of proton export transporters may be increased in cancer cells and that these transporters function to prevent lethal intracellular pH decreases as tumors grow and become more acidic. In a recent microarray screen, we noted differential expression of MCT1/SLC16A1 mRNA but not of any other SLC16A family members in relation to tumor phenotype (Wang et al., 2006). We therefore hypothesized that expression of one or more of the monocarboxylate transport proteins would be associated with more malignant subsets of neuroblastoma because these are the tumors most likely to be subjected to great pH stress. We further suggest that phar-

ABBREVIATIONS: MCT, monocarboxylate transporter; PCR, polymerase chain reaction; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; C.SNARF-1AM, carboxyl semi-naphthyl rhodofluor-1 acetoxymethyl ester; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; Q-RT, quantitative real-time RT-PCR; RT-PCR, reverse transcription; ELISA, enzyme-linked immunosorbent assay; MYCN, transcription factor mycN; NB, neuroblastoma.

macologic manipulation of these transport proteins is feasible and that the inhibition of such proteins may define a new class of novel therapeutics for neuroblastoma, and perhaps other neural crest-derived malignancies, such as melanoma (Balazs et al., 2001; Wahl et al., 2002).

Materials and Methods

Cell Lines and Primary Tumors

The neuroblastoma cell lines IMR32, Sk-N-SH, and NGP were obtained from the American Type Culture Collection (Manassas, VA). CHP134 cells were isolated from a patient at the Children's Hospital of Philadelphia. Rho-negative SY5Y neuroblastoma cells were generously provided by Dr. W. Davis Parker, Jr. (University of Virginia School of Medicine, Charlottesville, VA).

Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 26 mM sodium bicarbonate. Media were supplemented with 2 mM glutamine and 1% penicillin/streptomycin. Cells were maintained in a humidified 37°C incubator at 5% CO_2 for a pH of 7.3 or 15 mM sodium bicarbonate and 17% CO_2 to lower extracellular pH to 6.5. All of these cell lines were maintained in logarithmic growth at 37°C as monolayers in 75-cm² flasks.

Eighty-three primary neuroblastomas were obtained at diagnosis from children enrolled in Children's Oncology Group (COG) Biology studies. Patient clinical characteristics and risk group were determined using standard COG algorithms as described previously (Maris, 2005). Biopsy samples were snap-frozen in liquid nitrogen, and a diagnosis of neuroblastoma was confirmed by central pathologic review. *MYCN* gene copy number status was determined by fluorescence hybridization in situ; DNA index was determined by flow cytometry after cell dissociation (Look et al., 1991; Matthay et al., 1999). We estimated the percentage of tumor cell content in pilot sections before nucleic acid extraction. Only samples with a greater than 70% neuroblastoma cell content were included in this study.

We isolated RNA from exponentially growing cell lines or from 50 to 100 mg of snap-frozen tumor samples using TRIzol reagent (Invitrogen, Carlsbad, CA). All RNA samples were subjected to DNase I (Ambion, Austin, TX) digestion to eliminate contaminating genomic DNA, then purified with the RNeasy Mini Kit (QIAGEN, Valencia, CA). RNA quality was checked by spectrophotometry, 1% agarose gel, and by microfluidics-based electrophoretic analysis (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA). The Children's Hospital of Philadelphia Institutional Review Board approved this study.

Reagents

We obtained the fluorochrome Carboxy SNARF-1 acetoxymethyl ester (C.SNARF-1AM) and the detergent Pluronic F127 from Invitrogen. Matrigel was purchased from BD Biosciences (San Jose, CA). The MTS kit was obtained from Promega (Madison, WI). All PCR reagents except Tri Reagent were from Invitrogen. We obtained Tri Reagent and all other reagents, including lonidamine and α-cyano-4-hydroxy cinnamate, from Sigma Chemical Co. (St. Louis, MO). Lonidamine was diluted into media from a 16 mM stock solution of DMSO. DMSO and other vehicle controls were negative with respect to effects on intracellular pH and viability.

Western Blot Materials

Primary antibodies to MCT1 (C-20) and MycN (H-50) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody IR dye conjugates donkey anti-goat IR Dye 800 nm and goat anti-rabbit Alexa Fluor 680 nm were obtained from Rockland (Gilbertsville, PA) and Invitrogen, respectively. Cell Death Detection ELISA Plus 10x was obtained from Roche Applied Science (Indianapolis, IN). MYCN siRNA was obtained from Ambion. siRNA [sense strand, 5′-GGAGCAUGUUUUGUAUACAtt-3′; antisense strand, 5′-UGUAUACAAAACAUGCUCCtc-3′] and Silencer Negative Control

siRNA was also obtained from Ambion. Lipofectamine 2000 was obtained from Invitrogen.

Western Blotting Methods

Neuroblastoma cells were solubilized in aqueous buffer containing 2% SDS, 62.5 mM Tris, and 20% glycerol at pH 6.8, scraped from T-25 cell culture flasks, and placed in microcentrifuge tubes. Cells were pulsed for 5 s with a probe sonicator, vortexed for 10 s, and centrifuged at 1000g for 2 min. The protein levels of the samples were measured by BCA assay (Bio-Rad Laboratories, Hercules, CA). Samples were all standardized to 0.5 mg/ml protein by dilution with gel loading buffer containing 2% SDS, 62.5 mM Tris, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromphenol blue at pH 6.8. Samples were heated in boiling water for 5 min and then centrifuged at 1000g for 5 min. Each sample (20 μl) was loaded into each well of a 4 to 15% polyacrylamide Tris-HCl Bio-Rad Ready Gel for a total of 10 μg of protein per lane. Polyacrylamide gel electrophoresis was performed at 180V constant voltage in Tris/glycine/SDS running buffer (25 mM/192 mM/0.1%, respectively), pH 8.3, for 1 h. Protein was transferred to Hybond-C Extra Nitrocellulose 45 μm (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 30 min at 15-V constant voltage on a Bio-Rad Transblot Semi-dry transfer cell in Tris/glycine buffer (25 mM/192 mM) containing 10% methanol at pH 8.3. Membranes were blocked in Rockland blocking buffer for near-infrared fluorescent Western blotting at room temp for 1 h. Primary antibody was added at a dilution of 1:1000 in blocking buffer and incubated with the blots overnight at 4°C. Membranes were washed five times for five min in PBS with 0.05% Tween 20, after which the membranes were placed in blocking buffer with secondary antibody IR dye conjugate at a dilution of 1:10,000. Membranes were again washed five times for 5 min in PBS with 0.05% Tween 20 after which they were rinsed once with PBS. Western blots were read on a Li-cor Odyssey Infrared Imager (Lincoln, NE).

Primary Tumor Real-Time Quantitative RT-PCR. We used Q-RT-PCR to investigate expression of MCT1/*SLC16A1* in 83 primary neuroblastoma samples. One microgram of total RNA was used for cDNA synthesis with Superscript II reverse transcriptase (Invitrogen). Q-RT-PCR was performed using an ABI Prism SDS-7900HT thermal cycler (Applied Biosystems, Foster City, CA) in two separate reactions with differently labeled *MCT1* and glyceraldehyde 3-phosphate dehydrogenase. We obtained RNA-specific primers and probes from ABI "Assay-on Demand" (catalog number Hs00161826m1 for *MCT1*). Relative expression of target genes was determined by normalization to *GAPDH* according to the manufacturer's instructions. All Q-RT-PCR experiments included a no-template control and were performed in triplicate.

RT-PCR on Established Cell Lines. Three neuroblastoma cell lines, IMR32, Sk-N-SH, and NGP, were cultured at pH 6.5. Extracellular pH was adjusted by raising the CO_2 to 17% for 48 h; cells were then harvested, and total RNA was extracted by using Tri Reagent according to the manufacturer's instructions. RT-PCR was then performed. In brief, reverse transcription was carried out using the cloned AMV cDNA Synthesis Kit (Invitrogen) with random hexamers (25°C for 10 min followed by 50°C for 50 min). For the PCR reaction, Platinum *Taq* polymerase was used. The sequences of specific primers were as follows: MCT-1: sense, 5′-TTTCTTTGCGGCT-TCCGTTGTTG-3′; antisense, 5′-TCAATTTACCCTTCAGCCCCAT-GG-3′; MCT-4: sense, 5′-TTTTGCTGCTGGGCAACTTCTTCTG-3′; antisense, 5′-TCACGTTGTCTCGAAGCATGGGTTT-3′; MYCN: sense, 5′-CACAAGGCCCTCAGTACCTC-3′; antisense, 5′-GATCAG-CTCGCTGGACTGAG-3′; β-actin: sense, 5′-TGCGTGACATTAAGG-AGAAG-3′; antisense, 5′-CTGCATCCTGTCGGCAATG-3′.

The cycling parameters are as follows: for MCT1 and MCT4, 35 PCR cycles of 30 s for denaturing at 94°C, 30 s for annealing at 62°C, and 45 s for DNA synthesis at 72°C; for MYCN, 40 PCR cycles of 30 s for denaturing at 95°C, 30 s for annealing at 55°C, and 1 min for DNA synthesis at 72°C; for β-actin, 25 PCR cycles of 15 s for denaturing at 94°C, 30 s for annealing at 55°C, and 1 min for DNA

synthesis at 68°C. PCR products then underwent electrophoresis on ethidium bromide-stained 2% agarose gels.

Intracellular pH Measurements

Cell Preparation. Cells were plated on coverslips affixed to microwell dishes at a density of 0.2×10^6 cells in 2 ml of medium, 24 to 48 h before experiments. Coverslips were coated before plating with a 1:3 dilution of Matrigel in serum-free medium as described previously (Wahl et al., 2000). Preliminary experiments (data not shown) demonstrated that dye leakage during an experiment of 1 h or less was undetectable under these conditions.

Dye Loading. Cells were incubated for 15 min with 9 μM C.S-NARF-1AM (Owen et al., 1997) in medium containing 10% fetal bovine serum in a 37°C incubator under 5% CO_2 as described previously (Wahl et al., 1997, 2000). After a change of medium, the cells were further incubated for 20 min at 37°C, 5% CO_2, to complete hydrolysis of the dye ester to C.SNARF. Each plate was then mounted on the microscope stage and warmed to 37°C under flowing humidified air containing 5% CO_2. In some experiments, extracellular pH was lowered, which was accomplished by lowering the bicarbonate concentration in the medium while keeping the sodium concentration constant.

Fluorescence Microscopy and Calibration. Intracellular pH values were obtained from intracellular whole emission spectra of the pH-sensitive dye C.SNARF-1 (cleaved from the membrane permeable AM form of the dye). The dye was loaded into cells before mounting them on an inverted fluorescence microscope. The whole-spectrum approach to measurement, calibration technique, and experimental methodology had been described in detail (Wahl et al., 2002).

Protocol for Extended Intracellular pH Time Courses. The experiments were performed on substrate-adherent cells at 37°C in complete growth medium. First, steady-state intracellular pH was determined of cells in 2 ml of complete growth medium. The initial steady-state intracellular pH at extracellular pH 7.3 was measured several times on a field of 8 to 15 cells. We then replaced the medium with medium containing lonidamine and/or having an extracellular pH of 6.5. We then measured the intracellular pH for 30 min, during which it equilibrated. The fluorescence signal used to measure intracellular pH was collected on a single field of cells for 5 of every 10 s during the course of experiments.

[3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Inner Salt] Assay. The MTS assay (Sladowski et al., 1993) was performed to determine effects of lonidamine on metabolic viability under conditions designed to match the experiments in which intracellular pH measurements were made. Cells were seeded in 96-well culture plates (10,000 cells/well); after overnight preincubation, cells were exposed to varying concentrations of lonidamine for 48 h. The MTS reagents were then mixed and added to quantitate in a colorimetric assay the number of viable cells present in each treatment group. Color development was monitored with a spectrophotometer at 490 nm. The effect of lonidamine on metabolic viability was expressed as the percentage of cells that were viable relative to the percentage of such cells in untreated control cells.

Cell Death Assay. Roche Cell Death Detection ELISA Plus 10× was carried out according to the manufacturer's instructions for measuring both apoptotic cell death and necrotic cell death in adherent cells.

Statistical Analysis. Because distributions for gene expression data are skewed, rametric analyses were used. For the RT-PCR data and clinical correlations, the two-sample Wilcoxon rank-sum test was used to explore possible associations between MCT1 gene expression and age (dichotomized at 1 year of age), stage (4 versus other), risk group (high-risk versus other), Shimada histopathology (unfavorable versus favorable), DNA index (diploid versus other), and MYCN status (amplified versus not amplified). The Kruskal-Wallis test was used to examine gene expression with risk group of three categories. Intracellular pH and MTS data are expressed as means ± S.E. Student's t test was used to determine the significance between each experimental group. Box plots were used to describe gene expression by clinical and biological factors.

Results

MCT1/SLC16A1 mRNA Is Differentially Expressed in Neuroblastoma. Table 1 shows the distribution of clinical and biological covariates for the 83 neuroblastomas studied. Q-RT-PCR first showed high concordance with Affymetrix microarray information (Wang et al., 2006). High MCT1/SLC16A1 mRNA expression was significantly associated with age >1 year at diagnosis, stage 4 disease, unfavorable Shimada histopathology diploid DNA index, and MYCN amplification, and high-risk clinical group by COG criteria as shown in Fig. 1.

Neuroblastoma Cell Lines Exhibit MCT1 Message. Analysis of cell lines by RT-PCR at normal and low pH showed that MYCN and MCT1 were expressed in the IMR32 and NGP cell lines, both of which have MYCN amplification (Fig. 2). In contrast, Sk-N-SH cells with no genomic amplification of the *MYCN* locus, expressed low amounts of MCT1 and little MYCN. In NGP cells, MYCN and MCT1 were high at both normal and low extracellular pH. In all cases, MYCN expression and MCT1 expression were positively correlated.

Neuroblastoma Cell Lines Exhibit MCT Protein. Analysis of cell lines by Western blot indicates that MCT is expressed in all of the cell lines tested. Figure 3 shows similar expression of this protein in IMR32, NGP, and Sk-N-SH cells.

Lonidamine Dose Response during Acute Acidification of CHP134 Cells. Figure 4 shows the effect of lonidamine, an MCT inhibitor, on the intracellular pH of cells having an extracellular pH of 6.5. Lonidamine immediately exerted a lowering effect on intracellular pH, which reached a minimum level within 15 to 30 min. On continued exposure to lonidamine, a new steady-state intracellular pH was sus-

TABLE 1

Summary of Taqman PCR (MCT1/GAPDH × 10)

Variables	N	Median (Min, Max)	P Value
Age at diagnosis			0.033
<1 year	36	5.89 (3.24, 10.5)	
>1 year	47	7.25 (2.02, 14.4)	
Stage			0.0028
1, 2	24	5.30 (2.02, 9.55)	
3, 4	59	7.10 (2.17, 14.4)	
Stage			0.0009
1,2,3	43	5.65 (2.02, 10.1)	
4	40	7.63 (2.87, 14.4)	
Risk group			0.0016
Low	24	5.30 (2.02, 9.55)	
Intermediate	19	6.55 (2.17, 10.1)	
High and ultrahigh	40	7.96 (2.87, 14.4)	
Risk Group			0.0012
Low and intermediate	43	5.87 (2.02, 10.1)	
High and ultrahigh	40	7.96 (2.87, 14.4)	
Shimada			0.0032
Favorable	42	5.89 (2.02, 10.1)	
Unfavorable	39	7.76 (2.87, 14.4)	
DNA Index			0.028
Diploid	21	7.34 (2.87, 14.4)	
Hyperploid	57	6.05 (2.02, 12.4)	
MYCN			<0.0001
Amplification	16	9.46 (4.73, 14.4)	
Single Copy	67	5.91 (2.02, 10.1)	

tained. In this cell line at the highest dose of lonidamine, the intracellular pH decreased to below 6.5.

Lonidamine Lowered Intracellular pH at Low Extracellular pH. After extracellular acidification, intracellular pH was measured in IMR32, Sk-N-SH, and NGP cells after extracellular acidification combined with various concentrations of lonidamine to demonstrate a dose response (Table 2). All three cell lines showed concentration-dependent accentuation of the intracellular acidification achieved by lowering extracellular pH alone. However, the MYCN-amplified cell lines IMR32 and NGP showed more intracellular acidification. Similar results were obtained by incubating cells with
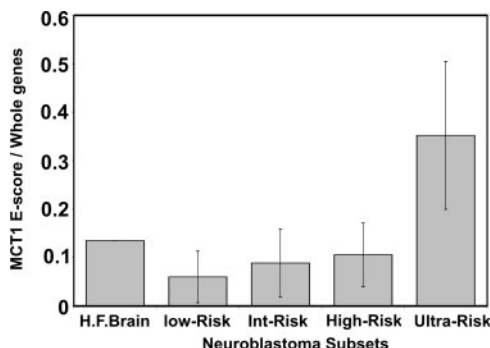

Fig. 1. Correlation of MCT1 mRNA expression with stage classification in neuroblastoma. The categories into which the NB biopsies were divided are as follows: human fetal (H.F.) brain (normal), low-risk, intermediate-risk (Int.), high-risk, and ultra-high-risk NB groups. These clinical categories are further defined as follows: low-risk NB were completely resectable, with no metastases. Moderate risk NB were *not* completely resectable (incomplete gross excision), with no metastases. High-risk NB were those with ipsilateral lymph node involvement. Ultra-high-risk NB were those with widely disseminated disease (common to liver, bone marrow, and/or skin and other organs).
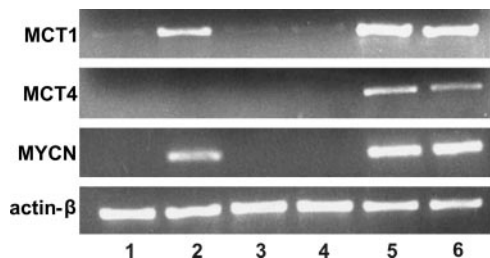

Fig. 2. Expression of MCT1, MCT4, and MYCN mRNA in three neuroblastoma cell lines by RT-PCR. Cells were cultured at pH 7.3 or 6.5 for 48 h, after which the cells were harvested and their total RNA was extracted for RT-PCR. Lane 1, IMR32 cells at pH 7.3; lane 2, IMR32 cells at pH 6.5; lane 3, Sk-N-SH cells at pH 7.3; lane 4, Sk-N-SH cells at pH 6.5; lane 5, NGP cells at pH 7.3; and lane 6, NGP cells at pH 6.5. The control in all lanes was β-actin. This experiment was repeated with similar results.
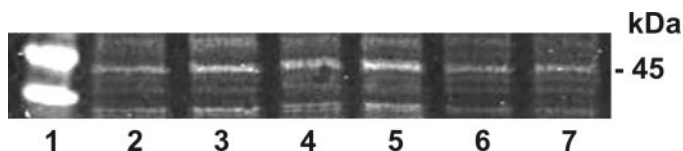

Fig. 3. Expression of MCT protein in three neuroblastoma cell lines. Cells were cultured at pH 6.5 or 7.3 for 48 h, after which the cells were harvested and their protein was extracted for Western blot. Lane 1, molecular weight standards; lane 2, Sk-N-SH cells at pH 6.5; lane 3, Sk-N-SH cells at pH 7.3; lane 4, NGP cells at pH 6.5; lane 5, NGP cells at pH 7.3; lane 6, IMR32 cells at pH 6.5; and lane 7, IMR32 cells at pH 7.3. The Western blot with anti-MCT1 is shown. This experiment was repeated with similar results.

increasing concentrations of extracellular sodium lactate at concentrations of 40, 80, and 160 mM (Table 2).

Lonidamine had no effect on intracellular pH when the extracellular pH was maintained at pH 7.3 (data not shown). When the experiments were repeated at an extracellular pH of 6.5, the intracellular pH of lonidamine-treated cells decreased significantly relative to the values achieved with extracellular acidification alone. For SK-N-SH cells, acute acidification to 6.5 caused intracellular pH to decrease from 7.41 ± 0.003 to 7.25 ± 0.003, a decrease of 0.16 pH. When lonidamine was added at 160, 240, or 320 μM, the net decreases in pH when combined with acidification were 0.21, 0.24, and 0.31 pH. For NGP cells, acidification alone decreased intracellular pH from 7.30 ± 0.07 to 7.01 ± 0.06, an average decrease of 0.23 pH. When lonidamine was added at 160, 240, or 320 μM, the net decreases in pH were 0.34, 0.52, and 0.55, respectively. For IMR32 cells, the initial intracellular pH of 7.47 ± 0.07 was decreased to 7.03 ± 0.05 by acidification to 6.5, giving an average decrease of 0.44 pH. When lonidamine was added at 160, 240, or 320 μM, the net decreases in pH were 0.83, 0.93, and 1.24 pH, respectively.

Comparing the results from these cell lines, we found that Sk-N-SH cells, which expressed negligible MCT1 (Fig. 3), showed the least decrease in intracellular pH after lonidamine treatment. IMR32 and NGP cells, which express higher levels of MCT1 (see Fig. 2), exhibited much greater decreases in intracellular pH under the same conditions, thus correlating the level of MCT1 expression with the lonidamine response.

Cell Line Metabolic Activity Was Compromised by Acidification and Lonidamine Administration. As shown in Fig. 5, lonidamine had a concentration-dependent cytotoxic effect in all cell lines studied. Compared with treatment at an extracellular pH of 7.3, lowering the extracellular pH to 6.5 induced a significant decrease in metabolic activity with increasing lonidamine concentration.

The results of using increasing concentrations of extracellular lactate or lonidamine in separate experiments measuring intracellular pH are shown in Table 2. When cells were incubated with 10 to 160 mM lactate, the effects on intracel-
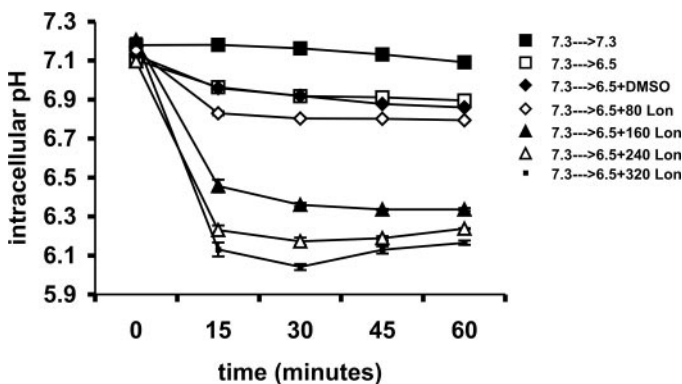

Fig. 4. Intracellular pH dose response to lonidamine with and without acute acidification in CHP134 cells. Cultured cells were subjected to increasing doses of lonidamine at pH 6.5. A dose response was observed from 80 to 320 μM. Intracellular pH was lowered to 6.5 or lower from 160 to 320 μM. ■, untreated. □, acute acidification to 6.5. ◆, acute acidification plus DMSO vehicle. ◇, 80 μM lonidamine plus acute acidification. ▲, 160 μM lonidamine plus acute acidification. △, 240 μM lonidamine plus acute acidification. Small ■, 320 μM lonidamine plus acute acidification.

lular pH were virtually identical to those of lonidamine, as well as to those of α-cyano-4-hydroxy cinnamate, an established MCT inhibitor (Dimmer et al., 2000) that has been shown to produce this effect in vitro in human melanoma cells (Wahl et al., 2002). These results support the hypothesis that the effect of lonidamine on intracellular pH is to reverse the gradient of lactic acid so that it becomes inwardly directed, causing an immediate and precipitous decline in intracellular pH, followed by decreased metabolic viability.

To determine whether the sudden decline in intracellular pH after lonidamine treatment was sufficient to cause cell death, the Cell Death Detection ELISA was performed. Figure 7 shows that both IMR32 and Sk-N-SH cells undergo apoptotic death in a dose-dependent fashion when subjected to lonidamine treatment at pH 6.7. An increase in absorbance at 405 nm corresponds to an increase in the concentration of nucleosomes, complexes of fragments of DNA and histones, in the cytoplasm of cells lysed in the apoptosis assay. IMR32 and Sk-N-SH neuroblastoma cells showed 3- and 4-fold increases in nucleosome concentration, respectively, with 160 μM lonidamine treatment at pH 6.7. Enrichment in nucleosomes in the cytoplasm of cells takes place because DNA degradation occurs several hours before plasma membrane breakdown during apoptosis. Very little or no increase in cell death resulted from lonidamine treatment at pH 7.3. Lonidamine treatment at either pH had no effect on necrotic cell death.

Figure 8 shows the effects of acute acidification plus lonidamine on neuroblastoma cells lacking mitochondria. In these rho-negative cells, an intracellular pH increase follows the short-term extracellular pH decrease from 7.3 to 6.5. This increase was sustained during the time course of treatment. The dose response to lonidamine indicates that these cells are responsive to lonidamine despite their lack of mitochondria. This is not surprising, given that cells without mitochondria would, in any case, produce fewer metabolic protons than do cells with mitochondria. When lonidamine was administered, the intracellular pH in these cells decreased as a function of time in a manner similar to that in cells with mitochondria.

Discussion

We have demonstrated, using primary biopsies and cell lines, that MCT1 activity is highly associated with MYCN amplification in human neuroblastoma. Thus, as is the case with MYCN, MCT1 expression seems to correlate with high-risk disease features such as the presence of metastatic disease at diagnosis. However, MYCN is unlikely to be a good therapeutic target because it is a transcription factor affecting the activity of numerous proteins (Dang et al., 1999). This work supports the hypothesis that MCT1 may be a more useful therapeutic target. The functional assays of MCT activity show that inducibility of the gene at low pH or amount

TABLE 2
Acidification with and without MCT inhibitors in neuroblastoma cells

| Extracellular pH before and after Drug Addition	Intracellular pH		
	NGP Cells	Sk-N-SH Cells	IMR-32 Cells
7.3→7.3	7.30 ± 0.01	7.41 ± 0.01	7.47 ± 0.01
7.3→6.5 + vehicle	7.07 ± 0.06	7.25 ± 0.003	7.03 ± 0.02
7.3→6.5 + 160 μM Lonidamine	6.96 ± 0.02	7.20 ± 0.02	6.64 ± 0.01
7.3→6.5 + 240 μM Lonidamine	6.78 ± 0.03	7.17 ± 0.04	6.54 + 0.01
7.3→6.5 + 320 μM Lonidamine	6.75 ± 0.02	7.10 ± 0.02	6.23 ± 0.24
7.3→6.5 + 40 mM Sodium lactate	7.04 ± 0.02	7.22 ± 0.02	6.68 ± 0.06
7.3→6.5 + 80 mM Sodium lactate	6.95 ± 0.02	7.13 ± 0.02	<6.0
7.3→6.5 + 160 mM Sodium lactate	6.88 ± 0.02	7.08 ± 0.02	<6.0
7.3→6.5 + 1.25 mM CNCn			<6.0
7.3→6.5 + 2.5 mM CNCn			<6.0
7.3→6.5 + 5 mM CNCn			<6.0

CNCn, α-cyano-4-hydroxy cinnamate.
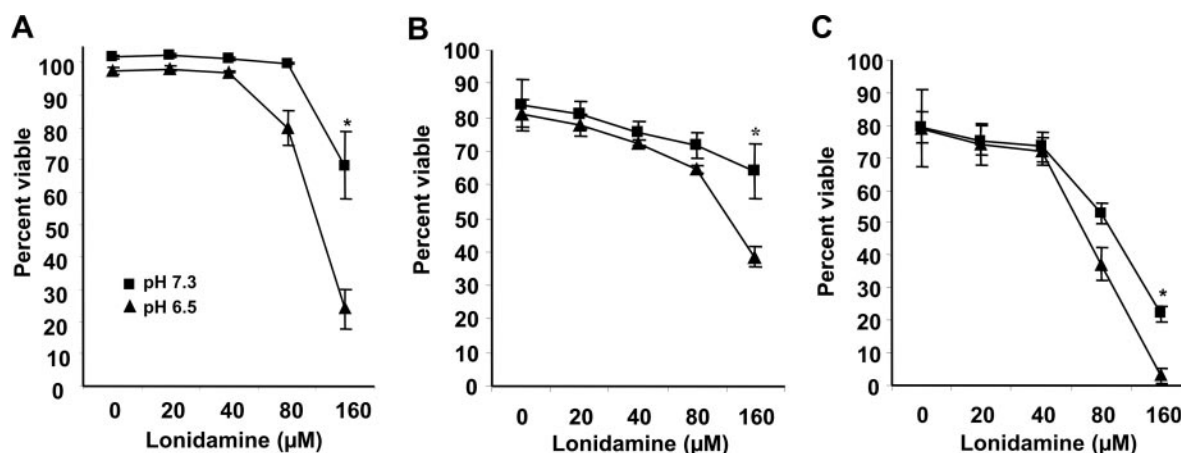
Fig. 5. A–C, cell viability as a function of lonidamine concentration. A, IMR32; B, Sk-N-SH; C, NGP. Cultured cells were subjected to increasing doses of lonidamine at pH 7.3 and 6.5 for 48 h. Viability was measured using the MTS assay. A dose response was observed from 20 to 160 μM. The percentage of IMR32 cells that died during exposure to 160 μM lonidamine at pH 6.5 was 23.9 ± 6.3%; the percentage of Sk-N-SH cells that died under the same conditions was 38.3 ± 3.0%.

of protein by Western blot are not predictive of how much an MCT inhibitor can affect intracellular pH. The findings indicate that inducibility as a function of extracellular pH was variable. The finding that the MCT1 can be constitutively on, off, or pH inducible is indeed a surprising finding. We think this may be indicative of variations in regulation of this gene. It may indirectly be affected by the relative amounts of oxidative aerobic metabolism versus anaerobic metabolism that varies within a tumor in space and time. It may also be due to the deregulation of normal signal transduction pathways, common to many forms of cancer. The experiments in which we attempted to knock-down MYCN and look at effects on MCT1 were inconclusive (data not shown), as were similar experiments performed on MYCN by Tang et al. (Tang et al., 2006).

Treating neuroblastoma cells with the MCT inhibitor lonidamine induced a dramatic and immediate decrease in intracellular pH (Fig. 3). This correlated with a significant loss of viability (Fig. 4) when extracellular pH decreased to 6.5, which is similar to the acidic microenvironmental pH of most tumors. MCT inhibitors can thus take advantage of the acidic pH tumor microenvironment to confer selectivity.

Our studies suggest that the mechanism by which sustained low intracellular pH can bring about cell death is primarily apoptosis. Watching closely as the cell medium was acidified and test compounds were added, we observed not only that the SNARF fluorescence per cell decreased 75% within the first 15 m of treatment but also that the cells did not detach and float. Dye leakage is an indication of compromised membrane integrity and viability, and impending karyolysis. We saw no signs of this from acute extracellular acidification without drug, which also did not cause any significant increase in apoptosis or necrosis by cell death ELISA.

The declines in intracellular pH to below 6.5 are significant in that intracellular metabolism ceases below 6.5; acidic endonucleases are activated and apoptosis can ensue (Eastman, 1995). In addition, all cellular enzymes have pH optimal for their activity, such that effects on the myriad of cytosolic and mitochondrial enzymes within the cells are certain to be multiple. This has led to considerable confusion in the literature regarding the identity of the lonidamine receptor. Another effect of lowering intracellular pH is the modification of protein folding in membranes, causing alterations in ligand binding to membrane-bound proteins.

Members of the MCT family of transporters are responsible for the export from cells of lactate, pyruvate, and a variety of other monocarboxylates (Dimmer et al., 2000). They are also referred to as organic anion symporters because they transport monocarboxylates in the same direction as protons. Therefore, we predicted that inhibition of these transporters could alter intracellular pH regulation, resulting in an inability of tumor cells to live in their acidic microenvironment.

To date, 14 MCT isoforms with various substrate specificities have been identified (Halestrap and Meredith, 2004). MCT1 is ubiquitous, with its highest levels found in skeletal muscle (Kim-Garcia et al., 1995). In most tissues, MCT1 is primarily a lactic acid transporter. The present study demonstrates that although MCT exchangers export lactic acid rather than regulate intracellular pH, inhibition of their activities can dramatically affect intracellular pH.

MCT1 gene expression has been reported in a few tumor cell lines other than neuroblastoma or melanoma (Lin et al., 1998), but MCT4 levels had not been measured in any tumors before our demonstration of elevated MCT1 and MCT4 activity in human melanoma cells grown at low extracellular pH (Wahl et al., 2002). The present study provides the first demonstration that the MCT transporters can be critical for maintaining intracellular pH in the viable range in neuroblastoma. Our results indicate that inhibitors of MCT could be useful adjuncts to neuroblastoma therapy, having particularly high activity where extracellular pH is low. Selectivity for the tumor microenvironment is based on the known low pH properties of the tumor. It has recently been reported that siRNA silencing of MCT in malignant glioma cells resulted in a precipitous decline in intracellular pH and cell death (Mathupala et al., 2004)

Lonidamine has been tested as an anticancer drug since the 1980s before much information was available regarding its mechanism of action (Di Cosimo et al., 2003; Oudard et al., 2003; Miyato and Ando, 2004). It was first reported that lonidamine inhibits lactate production in malignant gliomas but not in normal tissue, although the molecular mechanism for the lactate export was unknown (Paggi et al., 1988). Based on subsequent studies using magnetic resonance spectroscopy, lonidamine was proposed to be an inhibitor of lactic acid efflux that causes intracellular acidification (Ben-Horin et al., 1995). Increases in intracellular acidification in brain tumors were demonstrated. The present study provides in vitro support for these conclusions and extends the observation that inhibition of lactate efflux might be a therapeutically beneficial means of bringing about acidification to treat neuroblastoma.

Based on evidence from our own work and that of others (Floridi et al., 1981; Stryker and Gerweck, 1988; Ben-Horin et al., 1995; Pratesi et al., 1996; Pulselli et al., 1996; Ben-Yoseph et al., 1998; Mardor et al., 2000; Zhou et al., 2001), we propose that lonidamine is an inhibitor of MCT isoforms 1 and 4. Clinical studies testing the antitumor activity of lonidamine in adults found that its intravenous administration caused mild muscular and testicular discomfort (Scarantino et al., 1991), the tissues in which MCT1 and MCT4 isoforms are most prevalent (Wilson et al., 1998; Dimmer et al., 2000). When lonidamine was administered orally, however, there were no significant side effects, and an oral administration schedule is currently being studied in benign prostatic hyperplasia.

The present study, with its measurement of the immediate effect on intracellular pH, provides direct evidence of the entry of lactic acid into cells and inhibition of its export from cells subsequent to lonidamine treatment. Floridi et al. (1981) stated that micromolar amounts of lonidamine have major effects on plasma membranes. Our results support this statement, and the results shown in Figs. 5 and 6 strongly suggest that lonidamine-induced cytotoxicity is at least partly dependent on its inhibition of plasma membrane MCT1. The results shown in Fig. 7 show that the mode of cell death occurring during lonidamine exposure in IMR32 cells and Sk-N-SH cells is apoptosis as opposed to necrosis. Furthermore, as shown in Fig. 8, extracellular acidification of rho-negative (mitochondria-deficient) cells caused slight intracellular alkalinization as a result of the lack of proton production. These cells nev-

ertheless exhibited dose-dependent acidification in response to increasing concentrations of lonidamine in an acidic extracellular environment. Many types of tumors have high levels of lactate dehydrogenase (Koslowski et al., 2002; Kushner et al., 2003) and lactic acid (Walenta et al., 1997; Brizel et al., 2001). MCT1 and MCT4 have been found to be active at low pH in human melanoma cells (Wahl et al., 2002). No ion gradients are involved other than the gradients of lactate anions and the protons themselves for MCT activity. In a tumor with an acidic milieu, a lactate gradient could be used to drive H^+ transport against its transmembrane gradient, as originally described by Warburg (1930). The combined lactate and proton gradients provide the only requirement for transport into or out of the cell, because each requires the other to have electroneutral movement through the plasma membrane.

In conclusion, the results presented here indicate that MCT activity in neuroblastoma is high and correlates with high amplification of MYCN. Inhibitors of MCT have great potential when used to achieve selective compromise of neuroblastoma either by directly interfering with the viability of cells or improving the effectiveness of chemotherapeutic agents that function best at low extracellular pH, such as alkylating agents and platinum-containing compounds, but spare normal and surrounding cells at normal extracellular pH. Further exploration is clearly needed into what other tumor and cell types express high levels of MCT1 on their cell

surface and more importantly how the inhibition of MCT affects their cellular homeostasis. In addition, other considerations that pertain in vivo are the level of hypoxia and perfusion, which the present study did not address. Our study does indicate that extracellular pH can be a critical parameter of the tumor microenvironment for use in assess-
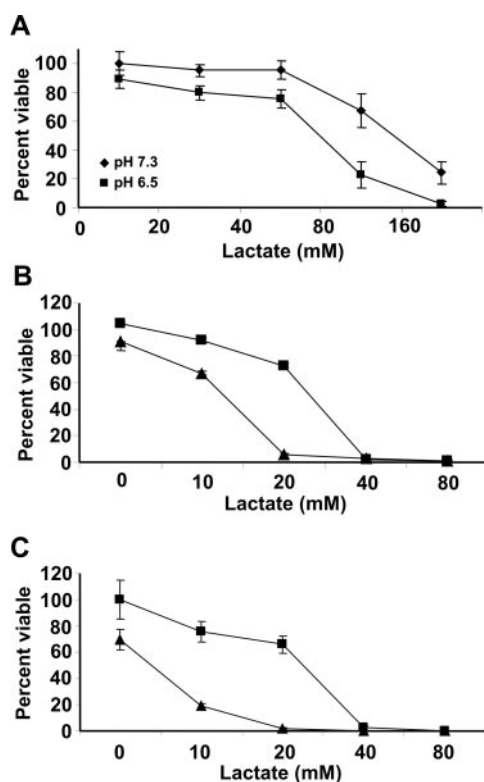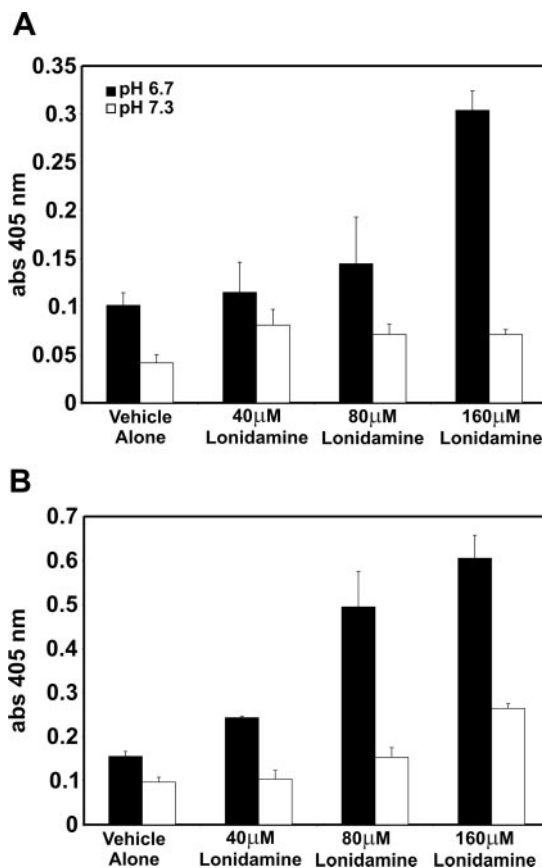
Fig. 7. Apoptosis assay of two neuroblastoma cell lines exposed to lonidamine and acidification. A cell death detection assay was performed by ELISA, which allows for determination of whether cytoplasmic histone-associated DNA fragmentation occurs after induction of cell death. This assay distinguishes apoptosis from necrosis. Cells were cultured at pH 6.7 or 7.3 and treated with vehicle or lonidamine for 24 h. Solid bars represent results in experiments performed at pH 6.7. Open bars represent experiments performed at pH 7.3. An increase in absorbance at 405 is indicative of increased cell death. A, IMR-32 cells. B, Sk-N-SH cells.
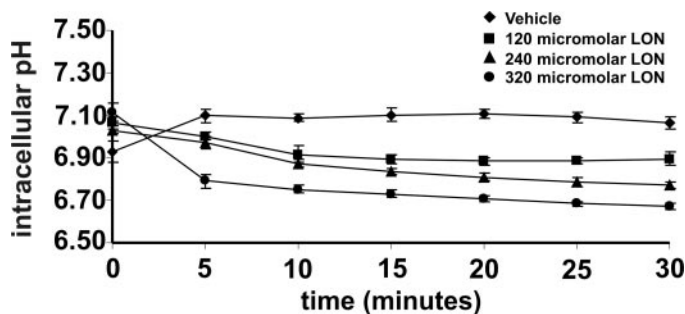
Fig. 6. A–C, cell viability as a function of lactate concentration. A, IMR32; B, Sk-N-SH; C, NGP. Cultured cells were subjected to increasing doses of lactate at pH 7.3 and pH 6.5. Viability was measured using the MTS assay. A dose response was observed from 10 to 80 mM. In all cell lines, there was a statistically significant difference between results obtained at pH 7.3 and pH 6.5; this was most pronounced in IMR32 cells (Fig. 4A). Data are means ± S.E. ∗, $P < 0.01$ ($n = 8$).

Fig. 8. Response of rho-negative Sy5Y cells to lonidamine when coupled with acute acidification. A steady-state intracellular pH was obtained, followed by acute extracellular acidification from pH 7.3 to 6.5 with or without lonidamine in a dose response. ♦, vehicle plus acute acidification; ■, 120 μM lonidamine with acute acidification; ▲, 240 μM lonidamine with acute acidification; ●, 320 μM lonidamine with acute acidification.

ing the potential effectiveness of therapeutic antitumor agents.

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