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Carbamoyl-phosphate synthetase 2 is identified as a novel target protein of methotrexate from chemical proteomics

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ABSTRACT : **Using agarose-coupled methotrexate, we have successfully isolated two proteins, which have strong interactions with methotrexate. The two proteins were analyzed by Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry and identified as carbamoyl-phosphate synthetase 2 and phosphoribosylglycinamide formyltransferase, respectively. Interestingly, both of these two proteins are essential key enzymes in nucleotide biosynthetic pathways, like dihydrofolate reductase, a well-known methotrexate target. We confirmed the specificity of their interactions between methotrexate and two target proteins by the methods of competition binding assay, which were followed by western blotting using antibody against carbamoyl-phosphate synthetase 2 and phosphoribosylglycinamide formyltransferase, respectively. Moreover, we could observe that carbamoyl-phosphate synthetase 2 is overexpressed in methotrexate-resistant MOLT-3 cells comparing with control MOLT-3 cells. This result indicates that carbamoyl-phosphate synthetase 2 may be a novel target of methotrexate in cancer therapy. We propose that chemical proteomics can be a powerful technique to identify target proteins of a chemical.**

Keywords : **methotrexate, chemical proteomics, MALDI-TOF mass spectrometry, and carbamoyl-phosphate synthetase 2**

Introduction

Methotrexate (MTX) is a well-known antifolate anticancer agent, which blocks regeneration of tetrahydrofolate by inhibiting dihydrofolate reductase enzyme. Folate metabolism has been a very popular target of drug development, which can be classified into two major drug groups, such as folate antagonists (e.g., methotrexate) and thymidylate synthase inhibitors (e.g., 5-fluorouracil). These agents are widely used in cancer chemotherapy, as treatment for rheumatoid arthritis (Ulrich *et al.*, 2002). Although MTX has been widely used for treating cancer such as acute leukemia and choriocarcinoma, there are some reports on MTX-mediated side effects (Bolla *et al.*, 1993; Bertin *et al.*, 1995). Antifolates, such as methotrexate, have a reputation for sporadic and unpredictable toxicity and are quite toxic because it kills rapidly replicating cells whether they are malignant or not (Takimoto, 1997). Stem cells in bone marrow, epithelial cells of the intestinal tract, and hair follicles are vulnerable to the action of methotrexate.

The identification of target proteins of a chemical (i.e., drugs or toxicants) has been a laborious and painful work in classical pharmacology and toxicology. The recent breakthrough in analytical methods such as Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry has allowed us to use chemical genomics technology in the target protein identification of chemicals. Generally, a ligand (either drug or toxicant) in its optimum condition should have high affinities to its target molecules for being pharmacologically or toxicologically effective to the target. Based on this basic biological principle, we have developed a chemical proteomic methodology for identifying biological targets of a chemical and for studying signal transduction. From the present study, we have identified carbamoyl-phosphate synthetase 2 as a novel target of methotrexate.

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Abbreviations: CAD, the multifunctional enzyme of carbamoyl-phosphate synthetase-aspartate transcarbamoylase-dihydroorotase; DHFR, dihydrofolate reductase; GART, the multifunctional glycinamide ribonucleotide synthetase-aminoimidazole ribonucleotide synthetase-glycinamide ribonucleotide transformylase; MALDI-TOF Mass Spectrometry, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry; MTX, methotrexate; TMQ, trimetrexate.

Materials and Methods

Materials

Methotrexate coupled agarose was purchased from Sigma (St. Louis, USA). Bio-Rad Silver Stain Kit was from Bio-Rad (Hercules, USA). Protease inhibitor mixture tablets were purchased from Roche Diagnostics (Easel, Switzerland). Rabbit polyclonal anti-CAD antibody (2188, recognizes CPS core fragment) was a generous gift from Dr. E. A. Carrey of Royal Free and University College Medical School, London, UK. Rabbit polyclonal anti-GART antibody (recognizes GARS-AIRS-GART) was a generous gift from Dr. D. Patterson of Eleanor Roosevelt Institute, Colorado, USA. All other chemicals were purchased from Sigma (St. Louis, USA). MOLT-3/TMQ200-MTX500 (200-fold TMQ-resistant and 500-fold MTX-resistant subline) and MOLT-3 (drug-sensitive parent cell line) were generous gift from Dr. Hayato Miyachi of Tokai University School of Medicine (Miyachi *et al.*, 1993).

U937 cell lysate preparation

Exponentially growing U937 cells were harvested by low speed centrifugation and washed with PBS. The cell pellet were treated with cell lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 50 mM NaF, 1 mM Sodium orthovanadate, 1 mM PMSF, 1 μg/ml leupeptin, 5 μg/ml aprotinin and 2 μM pepstatin A) and the cell lysates were vortexed vigorously and intermittently for 15 minute at 4°C. The lysates were centrifuged at 15000 rpm for 15 min at 4°C using a table-top centrifuge then the supernatant was transferred to a new eppendorf tube on ice. The protein concentration of the supernatant was determined by Bradford assay and used for chemical pull-down assay. The supernatant (5 mg/mL final) was incubated with an indicated amount of methotrexate-coupled agarose for 2 hr with continuous rocking in cold room.

Chemical pull-down assay

Methotrexate-coupled agarose was rinsed for three times with washing buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 50 mM NaF, 1 mM Sodium orthovanadate, 1 mM PMSF) just before use. For control, agarose (CL-4B) was prepared by the same method in parallel with the chemical-coupled agarose. The methotrexate-coupled agarose (methotrexate concentration is 50 μM at final unless otherwise indicated) as well as control agarose (CL-4B) were incubated with cell lysate (5 mg/mL/reaction) at 4°C for 2 hr. Non-specifically bound proteins to the beads were removed by rinsing them with washing buffer for three times, then SDS sample buffer was added to the beads and boiled for 5 minute at 95°C. Proteins bound to methotrexate with high affinities were separated by SDS-PAGE (6-16%), and then visualized by silver staining for MALDI-TOF mass spectrometry analysis.

Competition binding assay and elution of binding proteins

For validating the specificity of interaction between binding proteins and a chemical, we have employed competition-binding assay, which is generally used in receptor ligand binding study. Briefly, excess amount of free methotrexate (100x concentrations of coupled methotrexate) was added from the beginning of incubation for pull-down assay. A principle of competition binding assay is that the presence of excess amount of free (or unlabeled) ligand inhibits the specific interaction between a receptor and its bead-immobilized (or labeled) ligand by competition for the same binding site. For further verification of the binding specificity, we have performed the elution of binding proteins. Briefly, methotrexate-coupled agarose was incubated with cell lysate, and then rinsed with washing buffer for three times to remove all nonspecific binders. The beads were incubated in the presence of excess amount of free methotrexate (100x concentration of coupled methotrexate) for additional 2 hr at 4°C to elute high affinity binding proteins of methotrexate from the beads.

Protein Identification by Peptide Mass Fingerprinting Analysis

Fractions of the phenyl-Sepharose were subjected to SDS-PAGE. After stained with Silver staining, the candidate band was excised from the gel and digested with trypsin as described (Jensen *et al.*, 1996). A 1-μl aliquot of the total digest (total volume, 30 μl) was used for peptide mass fingerprinting (Mortz *et al.*, 1994; Shevchenko *et al.*, 1996). The masses of the tryptic peptides were measured with a Bruker REFLEX III time-of-flight mass spectrometer at Pohang University of Science and Technology. Matrix-assisted laser desorption/ionization was performed with α-cyano-4-hydroxycinnamic acid as the matrix. Trypsin autolysis products were used for internal calibration. Delayed ion extraction resulted in peptide masses with better than 50 ppm mass accuracy

on average. Comparison of the mass values against the Swiss-Prot database was performed using Mascot Search program (Matrix Science).

Immunoblotting

The initial sample preparation for immunoblotting was almost identical with silver staining. Briefly, after run on 6-16% SDS-PAGE, the separated proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) instead of silver staining. Blocking was performed with TTBS buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk. The membranes were then incubated with either rabbit polyclonal anti-CAD antibody or rabbit polyclonal anti-GART antibody for 4 hr at room temperature. Immunoblots were washed for 2 hr with changing TTBS buffer for every 20 minute, and then incubated with horseradish peroxidase-linked secondary antibody for 30 minute at room temperature. Immunoblots were washed for 1 hr with changing TTBS buffer for every 10 minute, and developed using horseradish peroxidase-dependent chemiluminescence (ECL) (Amersham Corp.).

Results and Discussion

Isolation of methotrexate binding proteins

Methotrexate is well known chemotherapeutic agent for treating hematological cancers. Hence, we have employed U937 human hematopoietic origin cell line for examining target proteins of methotrexate other than dihydrofolate reductase. From the chemical pull down assay with methotrexate-coupled agarose and silver staining, we could observe two major proteins, which strongly interacted with methotrexate, and we name them as p230 and p110, respectively (Fig. 1). The interactions were increased in (immobilized) methotrexate-dose dependent manner and saturated at 50 μM of MTX concentration. For confirming the specificity of the interactions, competition-binding assay was performed as described in Materials and Methods. The results showed that the interactions of p230 as well as p110 were almost completely disappeared in the presence of excess (100x) amount of free methotrexate, which was added from the beginning of the chemical pull-down reaction (Fig. 2). This suggests that the interactions are highly specific to methotrexate.

Identification of p230 and p110 proteins

Since p230 and p110 are specific binding partners of

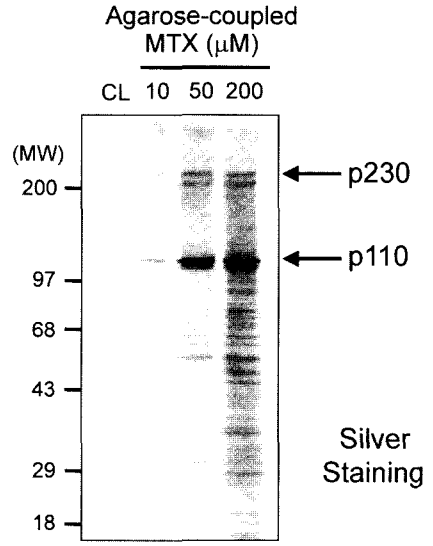

Fig. 1. Chemical pull-down of U937 cell lysate using agarose-immobilized methotrexate. Indicated concentrations of methotrexate-coupled agarose were incubated with U937 cell lysate at 4 for 2 hr as described in Materials and Methods. Briefly, after incubation with cell lysate, the agarose-coupled methotrexate resins were rinsed for three times with ice-cold washing buffer. The co-precipitated proteins were dissociated from the resins by adding SDS sample buffer and heating at 95 for 5 minute. The proteins were separated by run on 6-16% SDS-PAGE and visualized by silver staining. CL=CL-4B (control bead).

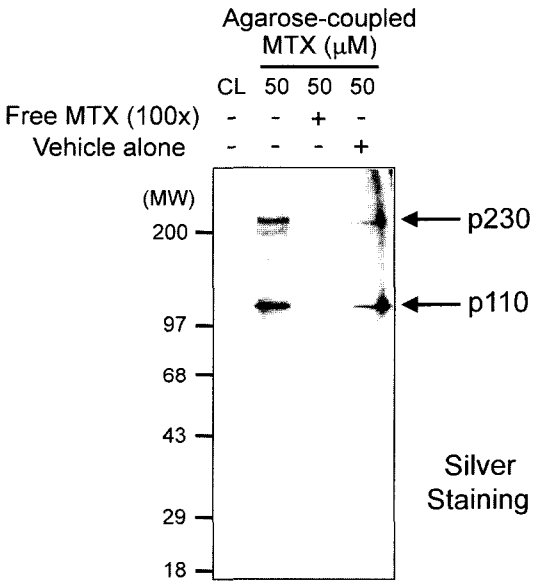

Fig. 2. Competition binding assay of protein-methotrexate interactions. Chemical pull-down assay was performed as described in Materials and Methods. Briefly, methotrexate-coupled agarose was incubated with cell lysate in the absence or presence of excess amount (100x concentration) of free (uncoupled) methotrexate. Sample preparation method for silver staining was almost identical as described in the legend of Figure 1.

methotrexate, we hypothesized these proteins could be possible targets of methotrexate. Therefore, the identities of p230 and p110 were analyzed using MALDI-TOF mass spectrometry and turned out as carbamoyl-phosphate synthetase 2 and phosphoribosylglycinamide formyltransferase. Carbamoyl-phosphate synthetase 2 is one of the components resided in a multifunctional enzyme CAD, which serves from the first to third step in pyrimidine biosynthesis (Raushel, 1998; Holden, 1999). On the other hand, phosphoribosylglycinamide formyltransferase is one of the components resided in a multifunctional enzyme GART, which serves the second, third, and fifth steps in *de novo* purine biosynthesis (Taylor, 1993). Considering DHFR, a purine biosynthetic enzyme, is a well-known target of MTX, it seems quite interesting that we have identified two key nucleotide biosynthetic enzymes, CAD and GART, as novel methotrexate targets in the present study. Dihydrofolate reductase is a well-known target enzyme of methotrexate in its cancer chemotherapy and has a molecular weight of about 21 kD. Although we could not observe a prominent protein band at the molecular weight of DHFR, we could detect the identity of DHFR from one of the several bands near the molecular weight by MALDI-TOF mass spectrometry analysis (data not shown).

Confirmation of CAD and GART by immunoblotting

To further confirm the identities of p230 and p110, we performed immunoblotting on methotrexate agarose precipitates after separating the proteins by SDS-PAGE. Based on the experiments combined with competition binding assay and elution from methotrexate agarose precipitates, we verified CAD as well as GART as strong binding proteins of methotrexate (Fig. 4). These results were highly consistent with those of MALDI-TOF mass spectrometry study. In fact, folate-dependent enzymes, including GART, have been reported to be regulated by antifolates, such as methotrexate analogues (Takimoto, 1997; Jackson, 1994; Costi, 2001). On the other hand, CAD has never been suggested as a target of antifolate drugs, suggesting it can be a novel target.

Higher expression of CAD in methotrexate-resistant MOLT-3 cells

As all the present data indicate that CAD and GART are possible target enzymes of methotrexate, we examined antifolate agents-resistant cell lines on the expression profiles of CAD and GART. MOLT-3 is a human acute

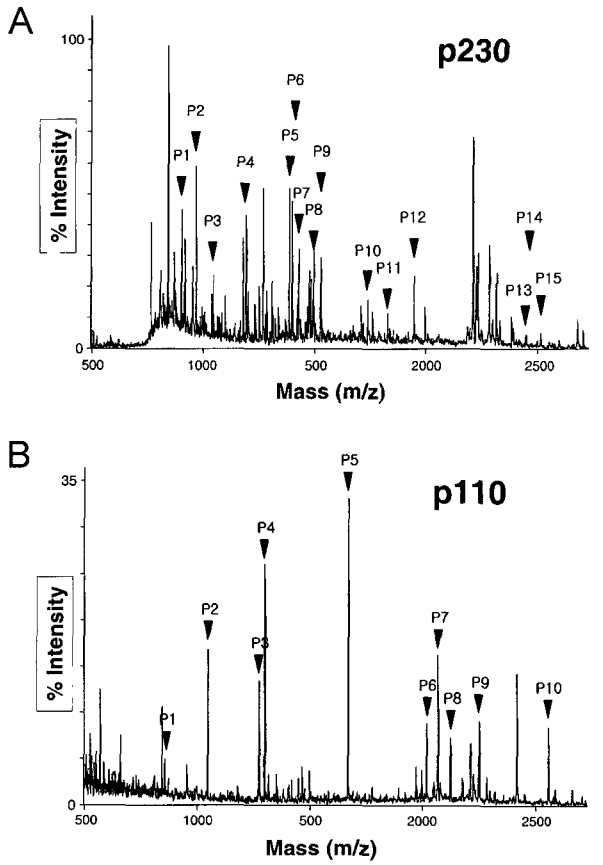

Fig. 3. Identification of p230 and p110 as CAD and GART, respectively. A) p230 isolated from proteins that co-precipitated with methotrexate was digested with trypsin, and the resulting peptide mixture was analyzed by MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry). Carbamoyl-phosphate synthetase 2 was identified with 10% coverage. The arrows (*P1-P15*) indicate matched peaks among the measured tryptic peaks of p230 with calculated molecular masses of Carbamoyl-phosphate synthetase 2 within 50 ppm. The results are shown in Table 1. B) p110 kD isolated from proteins that co-precipitated with methotrexate was also analyzed as described in the identification of p230. Phosphoribosylglycinamide formyltransferase was identified with 14% coverage. The arrows (*P1-P10*) indicate matched peaks among the measured tryptic peaks of p110 with calculated molecular masses of Phosphoribosylglycinamide formyltransferase within 50 ppm. The results are shown in Table 2.

lymphoblastic T-cell origin cell line. Up to now, other investigators have generated a number of MOLT-3 sublines, which show antifolate drug resistance, including methotrexate. Those cell lines are often reported to overexpress folate metabolic enzymes, such as DHFR, and those enzymes are presumably targets of the antifolate drugs (Ohnuma *et al.*, 1985; Miyachi *et al.*, 1993). Therefore, these cells

Table 1. Peptide sequences and masses from p230 by MALDI-TOF Mass Spectrometry

Peptide	Sequence	M + H$^+$	
		Observed	Calculated
		Da	
P1	YVAPPSLR (2103-2110)	901.46	901.502
P2	VPQFSFSR (1263-1270)	966.43	966.492
P3	MALLATVLGR (2215-2224)	1043.53	1043.616
P4	MALLATVLGRF (2215-2225)	1190.62	1190.684
P5	LYLNETFSELR (1557-1567)	1383.62	1383.703
P6	TPHVLVLGSGVYR (932-944)	1396.69	1396.782
P7	IIAHAQLLEQHR (843-854)	1427.73	1427.799
P8	QIALAVLSTELAVR (875-888)	1482.81	1482.876
P9	LLDTIGISQPQWR (1054-1066)	1525.77	1525.824
P10	IFVVAAALWAGYSVDR (810-825)	1736.88	1736.924
P11	YGNRGHNQPCLLVGSGR (271-287)	1826.97	1826.895
P12	VYFLPITPHYVTQVIR (450-465)	1945.07	1945.082
P13	AIVHAVGQELQVTGPFNLQLIAK (1184-1206)	2445.41	2445.373
P14	LAGADVVLGVEMTSTGEVAGFGESR (1271-1295)	2451.23	2451.194
P15	QEEFESIEEALPDTDVLYMTR (2126-2146)	2514.16	2514.146

Table 2. Peptide sequences and masses from p110 by MALDI-TOF Mass Spectrometry

Peptide	Sequence	M + H$^+$	
		Observed	Calculated[a]
		Da	
P1	VLAVTAIR(386-393)	841.50	841.538
P2	AIAFLQQPR(425-433)	1042.59	1042.592
P3	LGVDLDAQTWR(698-708)	1272.66	1272.646
P4	IYSHSLLPVLR(661-671)	1296.79	1296.755
P5	AFAHITGGGLLENIPR(677-692)	1664.95	1664.899
P6	VAVLISGTGSNLQALIDSTR(810-829)	2014.12	2014.105
P7	DIQQHKEEAWVIGSVVAR(752-769)	2064.09	2064.074
P8	IEFVVVGPEAPLAAGIVGNLR(67-87)	2120.22	2120.198
P9	KIEFVVVGPEAPLAAGIVGNLR(66-87)	2248.27	2248.293
P10	GVEITGFPEAQALGLEVFHAGTALK(351-375)	2554.30	2554.342

[a]Monoisotopic mass

can be a good source of screening antifolate drug targets by genomics approaches. In the present study, we have employed MOLT-3 (drug-sensitive parent cell line) and MOLT-3/TMQ200-MTX500 (200-fold TMQ-resistant and 500-fold MTX-resistant subline), and compared the expression profiles of CAD and GART between the two cell lines. Interestingly, we could observe CAD is highly overexpress in MOLT-3/TMQ200-MTX500 cells comparing with control MOLT-3 (Fig. 5). However, we could not detect any increase of GART expression in drug-resistant MOLT-3. Although we still need more proof on functional analysis, we propose CAD as a possible target of methotrexate analogues. It can be a very interesting study to examine the effects of various methotrexate analogues on CAD.

In conclusion, the chemical proteomics using agarose coupled methotrexate render us to identify two metabolic enzymes using MALDI-TOF analysis. They were turned out to be CAD and GART, key enzymes in pyrimidine and purine biosynthetic pathways, respectively. The identities of CAD and GART and their binding specificities to methotrexate were confirmed using various biochemical and pharmacological methods. The present results propose CAD as a possible novel target of methotrexate analogues.

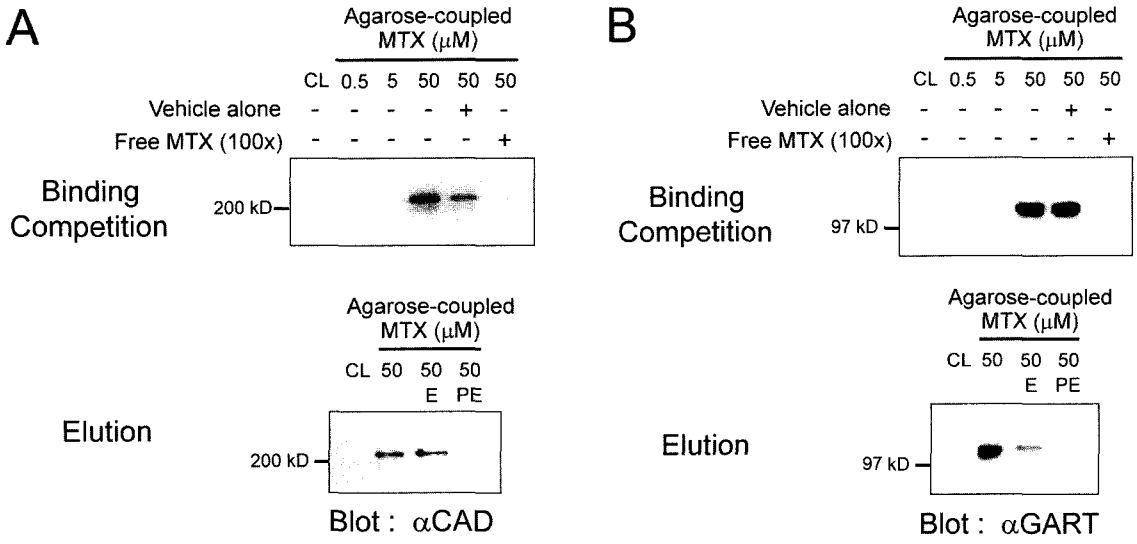

Fig. 4. Confirmation of mass spectrometry results by immunoblotting. A) For the determination of specificity of methotrexate-CAD interaction, competition binding assay was performed as described in Fig. 2 legend as well as in Materials and Methods. Then immunoblotting was performed using rabbit polyclonal anti-CAD antibody (*upper panel*). The specific interaction of methotrexate-CAD was yet once more confirmed by the elution of methotrexate-bound CAD as described in Materials and Methods. Briefly, methotrexate-bound CAD was almost completely eluted out into the elution buffer by adding 100-fold free methotrexate (E; elute) with negligible residual (PE; post-elute) (*lower panel*). B) The method of determining specificity of methotrexate-GART interaction was exactly same as for CAD of the above except using rabbit polyclonal anti-GART antibody. E=elute; PE=post-elute.

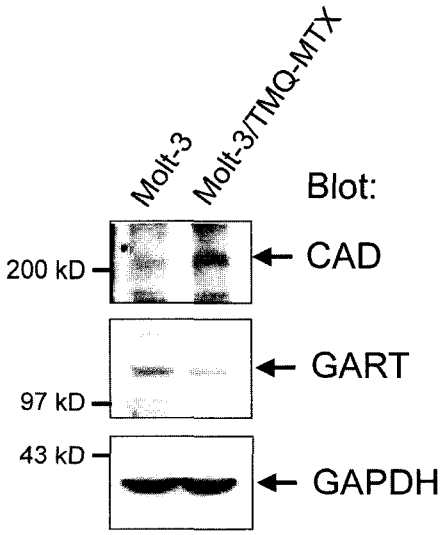

Fig. 5. Immunoblotting of CAD and GART in methotrexate-sensitive and methotrexate-resistant MOLT Cells. Exponentially growing MOLT-3 cells (drug-sensitive parent cell line) and MOLT-3/TMQ200-MTX500 (200-fold TMQ-resistant and 500-fold MTX-resistant subline) were harvested by low speed centrifugation and washed with PBS. The cell lysates were prepared and performed immunoblotting as described in Materials and Methods. For normalization of protein content in each cell lysate, mouse monoclonal anti-GAPDH antibody was used in immunoblotting as a control.

Acknowledgments

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