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Overexpression of sorcin in multidrug resistant human leukemia cells and its role in regulating cell apoptosis

Jing Qi [a,1], Ning Liu [b,c,1], Yuan Zhou [a], Yaohong Tan [a], Yanhong Cheng [a], Chunzheng Yang [a], Zhenping Zhu [a,*], Dongsheng Xiong [a,*]

[a] State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300020, China
[b] Laboratory of New Drug Research and Mass Spectrometry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, China
[c] Institute of Life Science, Jilin University, Changchun, China

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Abstract

In an attempt to identify novel proteins involved in the emergence of multidrug resistance (MDR) in leukemia cells, we adopted a proteomics approach to analyze protein expression patterns in leukemia cell lines, K562, and its MDR counterpart, K562/A02. Combining high resolution two-dimensional gel electrophoresis and mass spectrometry, we compared the protein expression profiles between K562 and K562/A02. A total number of 22 protein spots with altered abundances of more than 2-fold were detected and 14 proteins were successfully identified. Consistent with our previous observations by cDNA microarray, sorcin, a 22-kDa calcium-binding protein, was also identified by this proteomic approach with a 10.4-fold up-regulation in K562/A02 cells. Overexpression of sorcin protein in K562 cells by gene transfection led to significantly reduced cytosolic calcium level and increased resistance to cell apoptosis. Further, leukemia cell lines over-expressing sorcin also showed up-regulation of Bcl-2, along with decreased level of Bax. Taken together, our results suggest that sorcin plays an important role in the emergence of MDR in leukemia cells via regulating cell apoptosis pathways, thus may represent both a new MDR marker for prognosis and a good target for anti-MDR drugs development.

Keywords: Multidrug resistance; Sorcin; Bcl2/Bax; Apoptosis; Proteomics; 2D gel analysis

Multidrug resistance (MDR) in cancer, especially in leukemia, represents a major obstacle to successful chemotherapies [1–3]. Under experimental conditions, exposure of tumor cells to a single chemotherapeutic agent, e.g., doxorubicin, vincristine, or etoposide, can lead to development of resistance not only to the agent itself, but also to a broad range of structurally and functionally related and unrelated compounds. The mechanisms of MDR are polygenetic and yet to be completely defined [4–6]. Successful development of effective MDR reversal agents is, therefore, heavily dependent on our understanding to the various molecules/pathways involved in the emergence of MDR phenotype.

Two-dimensional gel electrophoresis (2-DE) is one of the most commonly used separation techniques in proteomics and is widely used in comparative studies of protein expression patterns in cells between different states [7]. Proteomics analysis, in combining both 2-DE and mass spectrometry (MS)-based methods, may provide solutions to the identification of post-translational modifications of cellular proteins, which would not normally be detected by gene analysis. Recently, 2-DE-based proteomic approaches have been utilized to study leukemia pathogenesis, identify new drug targets as well as drug toxicity [8–11]. Similarly approaches have been used to profiling protein expression patterns in cancer and in MDR cells in attempts to identify

* Corresponding authors.
E-mail addresses: qijing_cams@yahoo.com (J. Qi), zzhu.mab@gmail.com (Z. Zhu).
[1] These two authors contributed equally to this study.

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cellular proteins that are involved in tumorigenesis as well as those associated with resistance to specific drugs.

We have previously compared the gene expression profiles of K562 and its MDR counterpart, K562/A02 cells, using a cDNA microarray containing 1176 human genes [12–14]. K562/A02 cells have significant over-expression of P-glycoprotein (Pgp), the product of *mdr-1* gene, and are much more resistant to a variety of chemotherapeutic agents including doxorubicin, vincristine, and etoposide [12,15]. Out of a total of 12 differentially expressed genes identified, sorcin, a soluble calcium-binding protein gene, was shown to be associated with the emergence of MDR phenotype in human leukemia cells [14,15]. In the present study, in an attempt to further identify cellular proteins involved in the development of MDR in leukemia cells, we study the differential protein expression patterns between K562 and K562/A02, using 2-DE followed by MALDI-TOF MS analysis. A total of 22 differentially expressed protein spots were observed on the 2-DE gel, and 14 proteins were successfully identified following MALDI-TOF MS and database search. Consistent with our previous observations by cDNA microarray, sorcin was also identified by this proteomic approach with a 10.4-fold up-regulation in K562/A02 cells. Overexpression of sorcin protein in K562 cells by gene transfection led to significantly reduced cytosolic calcium level and increased resistance to cell apoptosis Further, leukemia cell lines over-expressing sorcin also showed up-regulation of Bcl-2, along with decreased level of Bax. These results provide definite proof that sorcin plays an important role in the development of MDR, likely via regulating the apoptotic pathways in leukemia cells.

Materials and methods

Cells and chemicals. The MDR-phenotype human leukemia cell line, K562/A02, was selected by exposure of K562 parental cells to stepwise increasing concentrations of doxorubicin [12]. K562/A02 and K562 cells were maintained in RPMI1640 medium (GIBCO, BRL Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) in the presence and absence of 1 μg/ml doxorubicin at 37 °C in a humidified 5% CO_2 atmosphere, respectively. All chemicals, including urea, CHAPS, Tris, DTT, bromophenol blue, and SDS were purchased from Sigma (St. Louis, MO).

2-DE analysis. Tumor cells were lysed in lysis buffer (8 M urea, 4% w/v CHAPS, 50 mM DTT, 25 mM spermine, 0.2% w/v Bio-Lyte pH 3–10) and centrifuged at 40000*g* for 60 min. The supernatant were collected and the protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA). Isoelectric focusing was carried out on Protean IEF Cell apparatus using pH 4–7 IPG gel strips (Bio-Rad) at 20 °C. After stained with colloidal Coomassie blue G-250, the gels were scanned with a high-resolution scanner (Umax 1120, UMAX Technologies, Inc.) and the gel images were analyzed using PDQuest software (Version 7.1.1; Bio-Rad) according to the protocols provided by the manufacturer. To accurately compare spot quantities between gels, a normalization based on the total density was applied for each gel.

MALDI-MS analysis and database search. Differential spots were excised from gels, washed, destained, and dried, followed by incubation in the digestion solution containing 50 mM NH_4HCO_3 and 0.1 g/L TPCK-trypsin for 12 h at 37 °C. The resulting peptides were extracted, concentrated, and mixed with saturated CHCA matrix solution. A volume (1 μl) of the mixture containing CHCA matrix was loaded on a 96×2 well hydrophobic plastic surface sample plate (Applied Biosystems, Foster City, CA) and air-dried. The samples were analyzed with Voyager DE STR MALDI-TOF Mass Spectrometer (Applied Biosystems). A peptides mixture containing Angiotensin I, ACTH(1–17) and ACTH(18–39) was used as mass standards for external calibration. Database search with the monoisotopic peptide masses was performed against the NCBInr or SwissProt database using the peptide search engine ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound), MASCOT (http://www.matrixsciece.com) and MS-Fit (http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm).

Production of sorcin-overexpressing K562 cell line, K562/8E. K562 cells were transfected with the previously described pcDNA3.1/sorcin vector [15] using lipofectamine 2000 (Invitrogen Life Technologies, Gaithersburg, MD). The transfected cells were selected under G418, and individual clones were analyzed for sorcin expression by immunocytochemistry staining using a goat anti-sorcin polyclone antibody (Santa Cruz Biotechnology, Santa Cruz, CA). A stable sorcin-overexpressing clone, K562/8E, was selected for further studies.

Effect of sorcin on intraocular calcium level and distribution. K562, K562/A02, and K562/8E cells were washed three times with D-Hanks' buffer, followed by incubation with 50 μM Fluo-3-AM at 37 °C for 30 min in dark. Confocal images were acquired with a TCS-SP2 MP confocal system (Leica, Mannheim, Germany). Fluo-3-AM was excited at 488 nm and emitted fluorescence was collected at 540 nm. Intracellular calcium levels were measured with a computer analysis program running on Leica software.

Effect of sorcin on etoposide-induced apoptosis. Cell apoptosis was measured using the annexin V-FITC apoptosis detection kit (Becton Dickinson, San Diego, CA) following the manufacturer's protocol. Briefly, the leukemia cells were incubated with etoposide at 20 μg/ml for 24 h, washed twice and resuspensed in 100 μl binding buffer, followed by staining with both 5 μl annexin V and 10 μl PI in dark at RT for 15 min. The percent of cell apoptosis was determined by flow cytometry (FACSCalibar, Becton Dickinson, San Jose, CA).

Western blotting analysis of expression of sorcin, Bcl-2 and Bax. Total protein was extracted from the leukemia cell lysates, loaded and separated on 12% SDS–PAGE gels. The proteins were then transferred onto nitrocellulose membranes (Hybond N, Pharmacia Biotech, Piscataway, NJ), and the amount of sorcin, Bcl-2, and Bax were detected with anti-sorcin, anti-Bcl-2, and anti-Bax (Santa Cruz Biotechnology) and visualized by ECL assay.

Results

Protein expression profiling in K562/A02 cells and K562 cells

The protein expression patterns between K562/A02 cells and its parent K562 were compared using PDQuest software (Version 7.1.1; Bio-Rad). Protein spots with greater than 2-fold difference in density between the two cell lines were scored when the differences were observed in three replicate gels from at least two independent experiments. Fig. 1 shows a pair of 2-DE images of proteins from the drug resistant cell line K562/A02 and the parent cell line K562. A total of 41 protein spots were mapped in pairs on K562 and K562/A02 gels and the identity of 36 spots were identified by tryptic digestion followed by MALDI-MS analysis (not shown). Detailed gel analysis revealed 22 reproducible protein spots with more than 2-fold change in density between the two cell lines, 6 of which were down-regulated and 16 were up-regulated in K562/A02. Close-up images of

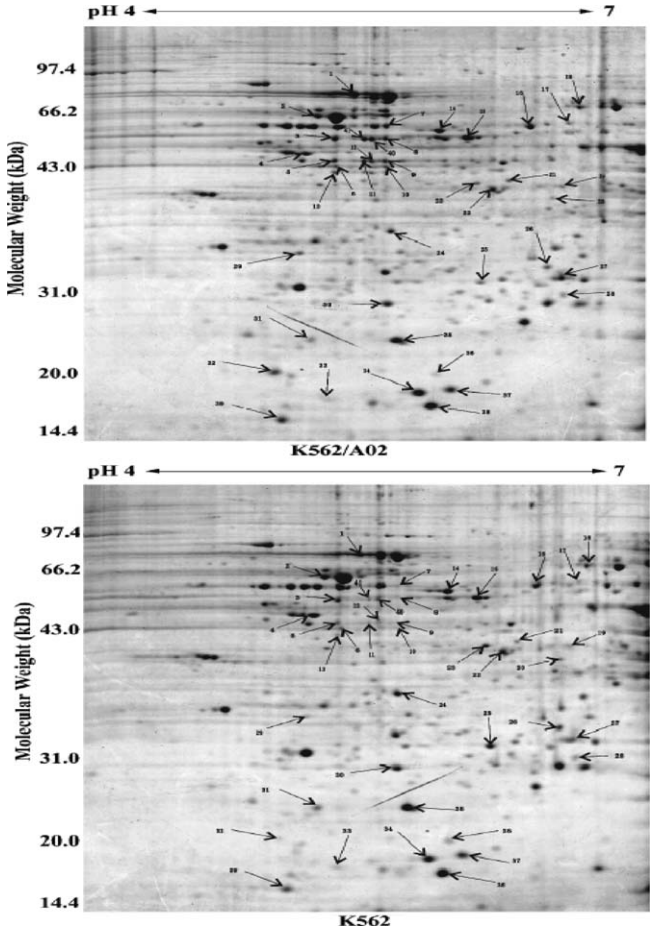


Fig. 1. Protein 2-DE maps of K562/A02 and its parent K562 cells. Gels were stained by colloidal Coomassie blue G-250.

the region of the 2-DE gels showing altered expression of a number of representative proteins in K562/A02 cells compared to their parent K562 cells is shown in Fig. 2. Of these 22 spots, 14 different proteins were identified by peptide mass fingerprinting out of 17 spots (there were 4 spots yielded the same protein, cytokeratin 18) (Fig. 2), and the rest five (spot 8, 13, 25, 40, and 41) yielded specific mass peaks with good resolution and signal/noise ratio but database search returned only low-quality candidates. As showed in Fig. 2B, the expression of sorcin (spot #32), vimentin (spot #7), cytokeratin 8 (spot #3) and cytokeratin 18 (spot #12) were significantly increased, whereas that of protein disulfide-isomerase (spot #14) and adapter-related protein (spot #33) were significantly down-regulated in K562/A02 cells. Further, there is evidence suggests that sorcin may undergo phosphorylation in K562/A02 cells—two possible phosphorylation sites, serine and threonine, were identified in one of the tryptic fragments of sorcin, SGTVDPQELQ KALTTMGFR (117–135aa), which corresponds to the peak with a molecular weight of 2254.02 as illustrated in Fig. 3. Phosphorylation of sorcin may play an important role in intracellular signaling.

Sorcin over-expression regulates expression of Pgp, Bcl-2, and Bax in leukemia cells

A sorcin over-expression cell line, K562/8E, was established by transfection of K562 cells with a pcDNA3.1/sorcin vector followed by G418 selection. Compared to the parent K562 cells, significant elevation of sorcin expression was detected in K562/8E cells by both immunocytochemistry (not shown) and Western blot assay (Fig. 4). It is interesting to note that Pgp was also slightly up-regulated in K562/8E cells (Fig. 4). Together with our observation that sorcin was up-regulated in MDR K562/A02 cells (Figs. 1 and 2), this result indicates that Pgp and sorcin are closely related and may regulate the expression of each other during the development of MDR. Sorcin over-expression also caused an effect in the expression of several apoptosis-related proteins, such as Bcl-2 and Bax. As shown in Fig. 4, the expression of Bcl-2 was significantly higher, whereas the level of Bax was moderately lower, in both K562/8E and K562/A02 cells than that in the parent K562 cells, leading to a greater Bcl-2 to Bax ratio in both K562/A02 and K562/8E cells.

Effect of sorcin on intracellular calcium level in leukemia cells

The effect of sorcin over-expression on the intracellular calcium concentration was examined in K562, K562/8E and K562/A02 cell lines. Both K562/8E and K562/A02 cells exhibited significantly lower intracellular calcium content than the parent K562 cells. Compared to K562 cells, the overall intracellular calcium concentration in sorcin over-expression cell lines, K562/A02 and K562/8E, was decreased by 88.4% and 58.5%, respectively.

Sorcin over-expression protects leukemia cells from etoposide-induced apoptosis

To examine the effect of sorcin on the chemotherapeutic agent-induced apoptosis, the sorcin-transfected K562/8E, the parent K562 and the MDR K562/A02 cells were incubated with etoposide for 24 h, followed by Annexin V/Propidium stain assay. As shown in Fig. 5, the apoptosis rates in leukemia cells treated with 20 μg/ml etoposide were 5.4%, 12.2%, and 3.6%, in K562/8E, K562, and K562/A02 cells, respectively. Together with its effects on regulating Bcl-2/Bax expression (Fig. 4), these results strongly suggest that sorcin may play an important role in regulating the apoptosis pathway in leukemia cells.

Discussion

The emergence of MDR is a major obstacle to effective leukemia treatment. A common mechanism of drug resistance to chemotherapy is the over-expression by tumor cells of one or more plasma membrane drug efflux proteins [16,17], such as Pgp, that function as efflux pumps to

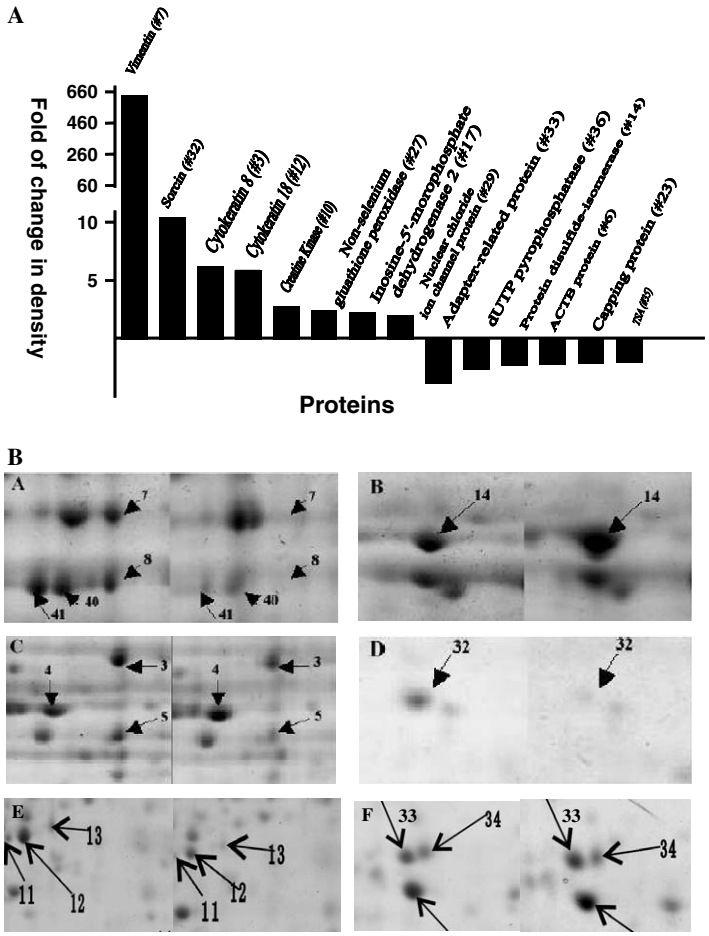
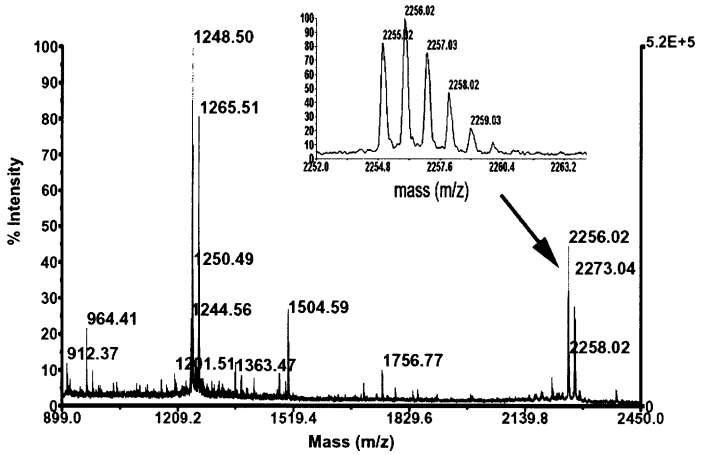


Fig. 2. (A) The differentially expressed proteins between K562/A02 and K562 cells as determined by 2-DE and MS analysis. The up-regulated proteins in K562/A02 cells are showed above the *X*-axis and the down-regulated proteins in K562/A02 are showed below the *X*-axis. Number in the parenthesis corresponds to that in Fig. 1 and panel B of this figure. (B) Close-up images of several regions of the 2-DE gels showing representative differentially expressed protein spots. Each panel (A–F) are representative pairs of images from the drug resistant cell line K562/A02 (left) and the parent cell line K562 (right). The spot numbers correspond to that in Fig. 1.

actively transport cytotoxic agents out of the tumor cells. Only limited clinical benefits have been achieved in the past years with various Pgp inhibitors, suggesting that simple inhibition of the "pumping" function of the efflux proteins may not be efficacious enough to completely reverse MDR in patients [18,19]. Further development of efficacious MDR reversal agents is, therefore, dependent on our complete understanding of the multiple molecular pathways/mechanisms involved in emergence of the MDR phenomenon.

In this study, in an attempt to identify novel protein molecules involved in the development of MDR in human leukemia cells, we analyzed protein expression patterns in K562 and its MDR counterpart, K562/A02, by proteomics analysis. A total number of 22 protein spots with altered abundances by more than 2-fold were identified and 14 proteins were successfully mapped by MALDI-MS and database search, including 8 up-regulation and 6 down-regulation in K562/A02 cells. These proteins belong to several groups based on their biological functions: (1) molecular chaperones, including cytokeratin 8 and cytokeratin 18; (2) proteins that function in cell motility or structure maintenance, such as vimentin; (3) proteins that are related to oxidation or reduction, such as non-selenium glutathione peroxidase; and (4) proteins involved in intracellular ion channel regulation, such as sorcin.

Sorcin was first identified in a vincristine-resistant Chinese hamster lung cell line, DC-3F/VCRd-5L [20], and later demonstrated to be over-expressed in over 50% of MDR cell lines, including those of human, hamster, and mouse origin, derived from selection against a variety of compounds such as colchicines, actinomycin D, taxol, vinblastine, teniposide, etoposide, and doxorubicin [21]. By gene profiling analysis we previously identified sorcin as one of the genes significantly up-regulated in the MDR cell line, K562/A02 [14]. Further, sorcin expression not only correlates directly to that of *mdr1* in primary human AML specimen, but also often indicates poor patient response to chemotherapies [14]. Under experimental conditions, over-expression of sorcin by DNA transfection into the non-resistant K562 cells conferred a MDR-like phenotype, which could be reversed by subsequent treatment with a



Measured Mass (Daltons)	Theoretical Mass (Daltons)	Residues #	Peptide sequence
911.36	911.45	128 to 135	ALTTMGFR (1)
963.40	963.49	77 to 84	LMVSMLDR
1200.50	1200.60	117 to 127	SGTVDPQELQK
1264.51	1264.58	107 to 116	QHFISFDTDR
1362.46	1362.56	85 to 96	DMSGTMGFNEFK
1378.47	1378.55	85 to 96	DMSGTMGFNEFK (1)
1503.59	1503.67	154 to 165	ITFDDYIACCVK
2254.02	2253.97	117 to 135	SGTVDPQELQKALTTMGFR (1) (2)
2271.03	2271.08	57 to 76	CLTQSGIAGGYKPFNLETCR

Fig. 3. (Top) MALDI-TOF mass spectrum obtained from spot #32 after trypsin digestion. (Bottom) Peptide sequences from sorcin (class 4 gene) matched with peaks obtained from the MALDI-TOF mass spectrum in (Top). (1) Oxidation site (methionine); (2) phosphorylation sites (serine and threonine).

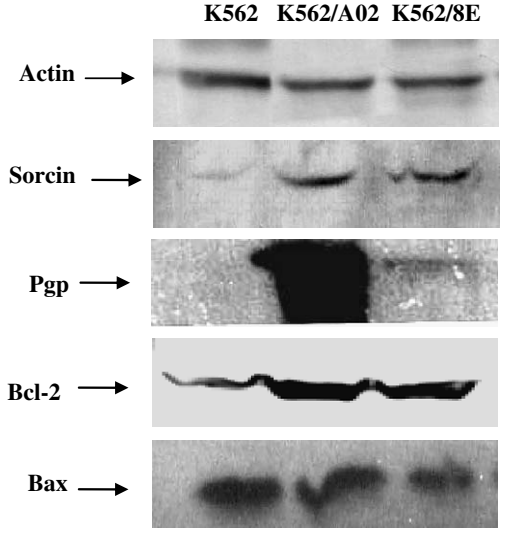


Fig. 4. Western-blot analysis of expression of sorcin, Pgp, Bcl-2, and Bax in K562, K562/A02, and K562/8E cells. Equal amount (100 μg proteins) of cell lysate proteins were separated by a 12% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blotted with antibodies to sorcin, Pgp, Bcl-2, Bax, or actin. All signals were detected using ECL detection system. Results are the representative of three similar experiments.

sorcin-specific siRNA [15]. Taken together, these observations suggest that sorcin may play an important role in the emergence of drug resistance and thus represent a significant and independent indicator of leukemia resistance to chemotherapies.

In order to elucidate the underlying mechanisms by which sorcin contributes to the emergence of drug resistance in leukemia cells, we established a sorcin overexpression cell line, K562/8E, by DNA transfection, and examined the effects of sorcin overexpression on both chemotherapeutic agent-induced apoptosis and the expression of apoptosis-related proteins in these cells. Similar to the MDR K562/A02 cells, K562/8E cells showed an up-regulation of Bcl-2 expression, while the level of Bax was moderately reduced, resulting in increased resistance to etoposide-induced apoptosis (Fig. 4). In addition, sorcin may also regulate cell apoptosis by modulating intracellular calcium level and/or distribution. As a soluble cytosolic calcium-binding protein, sorcin may sequester significant amount of cytosolic calcium. Further, sorcin may be translocated to the endoplasmic reticulum membrane and block calcium release mediated by the cardiac ryanodine receptor (RyR) [22,23]. Our result showing sorcin over-expression in leukemia cells leads to significantly reduced intracellular calcium concentration is in good agreement with the phys-

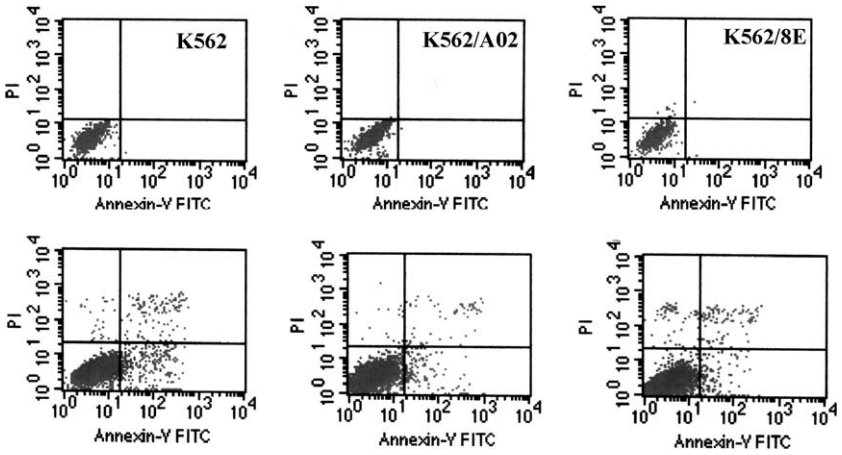


Fig. 5. Sorcin over-expression in leukemia cells results in increased resistance to cytotoxic agent-induced apoptosis. K562, K562/A02, and K562/8E cells were incubated with PBS (upper panels) or etoposide (lower panels) for 24 h, labeled with annexin V/propidium and analyzed by flow cytometry. Results shown are the representatives of two similar experiments.

iological functions of the molecule. It has been well documented that reduced intracellular calcium level contributes to the decreased sensitivity of cancer cells to cytotoxic agent-mediated apoptosis, leading to the development of drug resistance [24,25].

In conclusion, we here identified, by proteomic analyses, a panel of proteins that may be associated with drug resistance in leukemia cells, suggesting the development of MDR is a complex phenomenon possibly involving multiple mechanisms. We further confirmed the results of our earlier gene array study and showed that sorcin is up-regulated in MDR leukemia cells on both gene as well as protein levels. Finally, we demonstrated that sorcin may exert its activity in drug resistance via regulating the expression of apoptosis-associated proteins, such as Bcl-2 and Bax, and modulating intracellular calcium concentration and distribution. Taken together, these observations indicate that sorcin may represent not only a good diagnostic/prognostic marker but also a good target for modulation in our continuous efforts to searching and developing more efficacious MDR reversal agents.

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