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Immunocytochemical Identification and Localization of the Mr 22,000 Calcium-binding Protein (Sorcin) in an Adriamycin-resistant Myelogenous Leukemia Cell Line

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Monoclonal antibody against the Mr 22,000 calcium-binding protein (sorcin) from an adriamycin-resistant myelogenous leukemia cell line K562 (K562/ADM) was prepared and used as a probe to study the localization of sorcin in K562/ADM cells and the parental cell line, K562. Analysis of extracts from K562/ADM cells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorescence image analysis showed that K562/ADM cells possessed abundant sorcin in the cytoplasm which was almost entirely absent from the drug-sensitive parental cell line, K562. Furthermore, immuno-electron microscopic studies revealed that sorcin was closely associated with free ribosomes, rough endoplasmic reticulum, mitochondria, microfilament bundles and perinuclear membranes. These observations provide the first clue that the Ca-binding protein, sorcin, may play an important role in the development of the multidrug resistance phenomenon, although the relationship between sorcin and P-glycoprotein is still unknown.

Key words: Sorcin-Localization — Calcium-binding protein (22 kD) — Adriamycin-resistant K562

It has recently been shown that the adriamycin-resistant K562 cell line K562/ADM[5] possesses a 170–180-kD P-glyocoprotein on the membrane, displays resistance to various drugs including *Vinca* alkaloids, etoposide and colchicine,[1,2] and is considered to play an important role in drug efflux.[3] Furthermore, this 170–180-kD P-glycoprotein is highly expressed in other organs, including adrenal, kidney, placenta, and some untreated lung and breast cancers, and may have other physiological functions.[4,5] Besides the overproduction of P-glycoprotein in the plasma membrane, a calcium-binding protein (sorcin) has been found very recently in multidrug-resistant murine and hamster cells[6] and is homologous to calcium-binding light chain of calpain.[7]

As we have succeeded in preparing highly purified human sorcin and murine Mab against sorcin, the present experiments were designed to elucidate the localization of sorcin in K562 and K562/ADM cells. The paper first reports that sorcin is present only in cytoplasm and is closely associated with free ribosomes, rough endoplasmic reticulum (ER), mitochondria, microfilaments and perinuclear membranes, although there was a technical limitation in localizing this protein.

MATERIALS AND METHODS

Cell lines A human myelogenous leukemia cell line, K562 and the adriamycin-resistant K562 cell line, K562/ADM, were supplied by one of the authors.[1] They were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS)(Hyclone Laboratories, Logan, CA).

Preparation and characterization of monoclonal antibody against sorcin Murine monoclonal antibody (Mab) (HOT104) recognizing human sorcin was obtained by injecting crude membrane fractions from K562/ADM cells (10 μg protein per mouse) ip with complete Freund's adjuvant, fusing spleen cells from the immunized mice with P3-X63-Ag8-653-Ul myeloma cells, and cloning the resulting hybridoma cells secreting Mab against sorcin. The HOT104 Mab reacted with sorcin that had been highly purified by cell extraction of K562/ADM cells, ammonium sulfate fractionation, DEAE-Sephacel chromatography and gel filtration. The immunoglobulin subclass of HOT104 Mab is IgG1.[8]

Solubilization of K562 and K562/ADM cells K562/ADM cells were solubilized in diluted RIPA buffer [50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 2 m*M*

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[5] Abbreviations used in this paper: Mab, monoclonal antibody; K562/ADM, adriamycin-resistant K562; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PLP, periodate-lysine-paraformaldehyde; kD, kilodalton; ER, endoplasmic reticulum.

phenylmethylsulfonyl fluoride, containing 0.05% Triton X-100, 0.05% sodium deoxycholate, and 0.005% sodium dodecyl sulfate (SDS)].[9] The solubilized protein measurement was done as described elsewhere using bovine serum albumin (BSA) as a standard.[10]

Immunoblotting SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli.[9] The gels were fixed and stained with Coomassie brilliant blue. After SDS-PAGE, the proteins were transferred onto nitrocellulose sheets (0.45 μm, Bio-Rad Laboratories, Richmond, CA) by 3-h electrical transfer at 50 V in 25 m*M* Tris, 0.192 *M* glycine, pH 8.3, containing 20% (v/v) methanol. The nitrocellulose sheets were washed briefly with double-distilled water and incubated for 1 h with phosphate-buffered saline (PBS) supplemented with 5% BSA, and for 1 h at room temperature in TPBS (PBS containing 0.05% Tween 20) containing 10 μg/ml of HOT104 Mab. The sheets were then washed in TPBS for 10 min, three times. Thereafter, biotinylated horse anti-mouse IgGs (1:100 diluted, Vector Laboratories, Burlingame, CA) was added for 30 min at room temperature. After washing three times with TPBS, the sheets were treated for 30 min with diluted ABC reagent (avidin-biotin-peroxidase complex), and finally developed (5 min) in a solution freshly prepared by dissolving 4.5 mg of diaminobenzidine tetrahydrochloride (DAB)(Sigma Chemical Co., St. Louis, MO) in 20 ml of PBS, to which was added 10 μl of a 30% H_2O_2 solution just before incubation.[11]

Fluorescence image analysis (FIA) FIA was done, employing an Anchored-cell Analysis and Sorting (ACAS) 470 system (Meridian Instruments Inc., Okemos, MI). First, K562 and K562/ADM cells (5×10^6/ml) were reacted with 100 μl of HOT104 Mab (10 μg/ml) in PBS containing 3% BSA at room temperature for 30 min. After washing, the cells were reacted with FITC-labeled goat anti-mouse immunoglobulins (1:20 diluted, Coulter Clone) at room temperature for 15 min. After careful washing, they were mounted on an image scanner, and a laser beam of appropriate wavelength for exciting the FITC probe was directed through a microscope objective and focused in a fixed plane. The fluorescence of FITC labeling in the cells was registered as a function of position in a two-dimensional field. In this way, images of the fluorescing species could be reconstructed from the data. The cells were color-displayed based on the quantities of antigens recognized by the HOT104 Mab. At the same time, relative antigenic densities were obtained by scanning the FITC-positive cells in cut sections of the cells.[12,13]

Immunocytochemistry The confluent K562 and K562/ADM cells were fixed with periodate-lysine-paraformaldehyde (PLP) for 2 h at 4°C.[14] After careful washings with 20% sucrose in PBS, 10% sucrose in PBS, 5% sucrose in PBS and finally PBS, the cells were treated with PBS containing 0.5% saponin for 6 h at 4°C according to the method of Tougard and Picart with a slight modification.[15] After washing three times with PBS, the cells were treated with 10 μg/ml of HOT104 Mab at 4°C overnight. A negative control was prepared by incubating cells with normal mouse immunoglobulins (1:50 diluted, Sigma) overnight. They were then cultured with biotinylated horse anti-mouse IgGs (1:50 diluted, Vector Laboratories) for 3 h at 4°C. After rinsing with PBS, they were incubated with the diluted ABC reagent for 1 h at room temperature. The final color reaction was achieved by incubating the cells with 0.1% H_2O_2 and 0.05% DAB in 0.05 *M* Tris buffer, pH 7.2, for 5 min.[16] The cells were post-fixed with 2.5% glutaraldehyde in PBS, followed by 1% osmic acid, dehydrated with a graded ethanol series and embedded in Epon 812 resin. After polymerization at 60°C for 48 h, thin sections were made with an ULTRACUT E (C. Reichert Optische Werke AG, Vienna). Semi-thin sections were also prepared for optical microscopical examination. The thin sections were subsequently stained with uranyl acetate for 10 min and examined with an electron microscope (100C, Japan Electron Optics Laboratory, Tokyo).

RESULTS

Immunoblotting First, we examined by immunoblotting whether HOT104 Mab recognized sorcin in K562/ADM (K/A) cells. As shown in Fig. 1, HOT104 Mab reacted

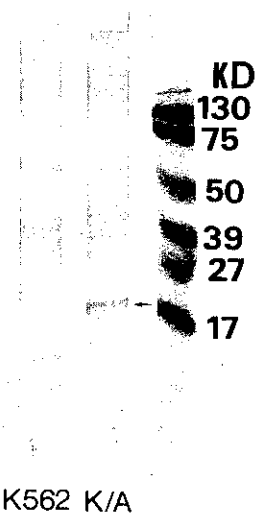


Fig. 1. Western blot analysis of the solubilized proteins (50 μg/lane) from K562 and K562/ADM (K/A) cells using HOT104 Mab. The right lane shows molecular size markers (in kD). A band corresponding to a protein of ca. 22 kD (arrow) is clearly seen in the lane for K562/ADM cells, but not in the lane for K562 cells. ABC-PO method.

with the solubilized protein with molecular weight of ca. 22 kD from K562/ADM cells, but not with the solubilized protein from K562 cells. Although weak, minor bands were seen in the lanes of both K562/ADM and K562 cells, they were found to be non-specific. After the solubilized protein from K562/ADM cells had been reacted with HOT104 Mab at room temperature for 1 h, the supernatant after centrifugation at 10,000 rpm for 30 min was collected, electrophoresed, and electrically blotted onto nitrocellulose membrane. The major band of ca. 22 kD after absorption reaction with HOT104 Mab was not found in the K562/ADM lane (data not shown).

Fluorescence image analysis (FIA) An Anchored-cell Analysis and Sorting (ACAS) system was employed to determine the localization and density of human sorcin. Figures 2a and 2b reveal that sorcin was present mainly in the cytoplasm. This observation was further confirmed by the finding that the relative density of sorcin was high in the cytoplasm, but low in the nucleus (Fig. 2c).

Immunocytochemistry As a further step, immuno-electron microscopy was utilized to determine the precise location of sorcin in K562/ADM cells. Figures 3a and 3b show that sorcin was predominantly present in the cytoplasm of K562/ADM cells, but not in K562 cells. Figures 4–7 show that sorcin was closely attached to free ribosomes, rough ER, mitochondria, perinuclear membranes and microfilaments. Although immunocytochemical examination by light microscopy revealed no sorcin in K562 cells (Fig. 3), immuno-electron microscopy showed that sorcin was stained positively but weakly with HOT104 Mab (Fig. 8a).

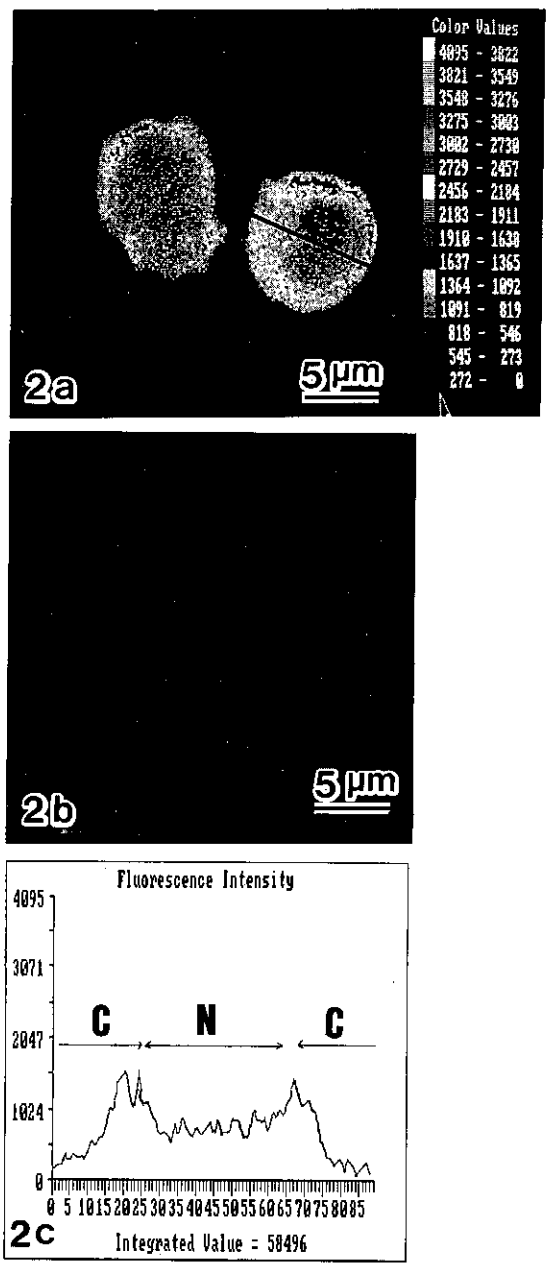


Fig. 2. Fluorescence image analysis of K562 and K562/ADM cells using an ACAS 470 system (×1,000). K562/ADM cells are clearly stained positively with HOT104 Mab (a), but K562 cells (b) are hardly stained. Relative sorcin density in a cut section of a K562/ADM cell is shown in 2a. Sorcin is present in the cytoplasm (C), but not in the nucleus (N) (c).

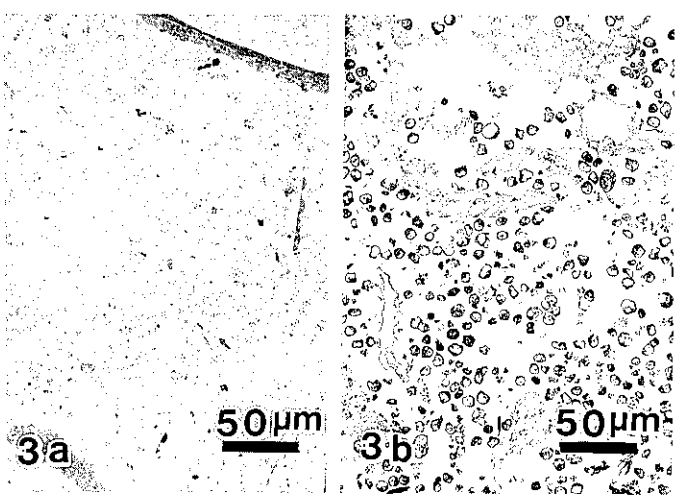
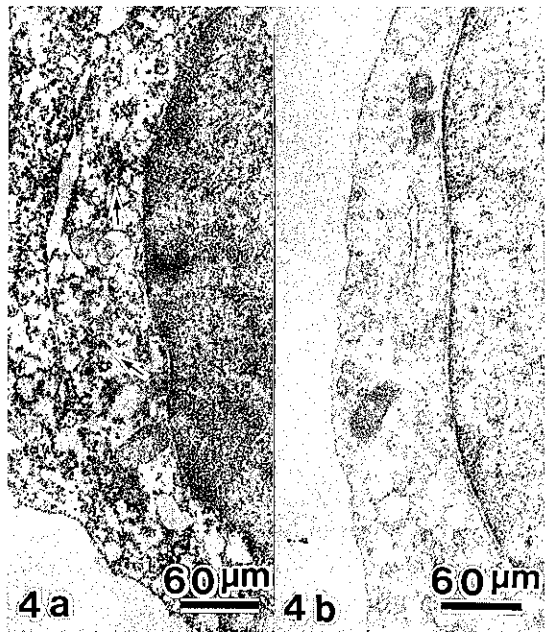
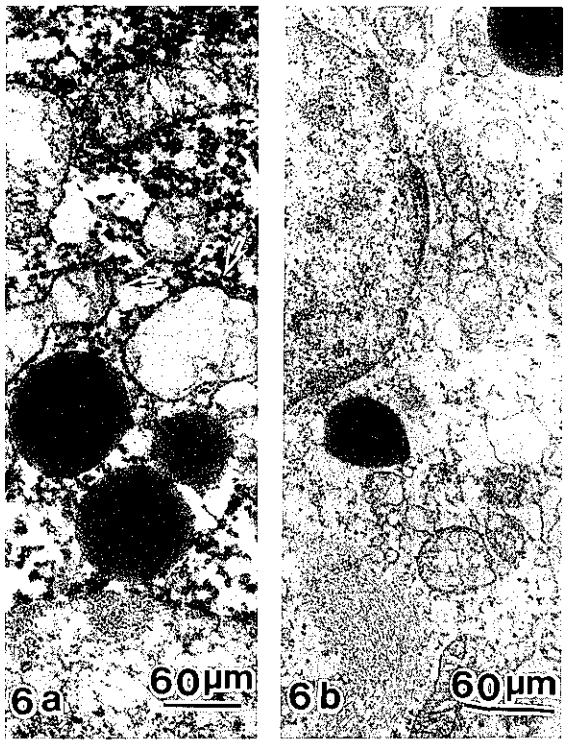
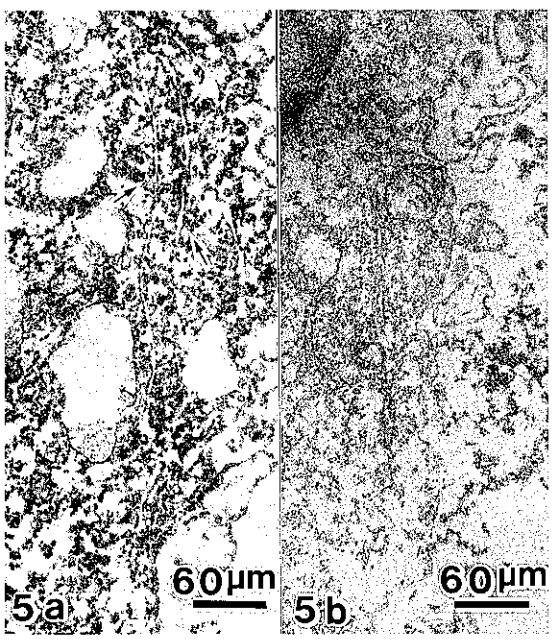


Fig. 3. Semi-thin sections from samples of K562 and K562/ADM cells embedded in Epon 812 after completion of immunocytochemical staining (×800). ABC-PO method. K562 cells are hardly stained positively with HOT104 Mab (a), but K562/ADM cells (b) are stained clearly and intensely.







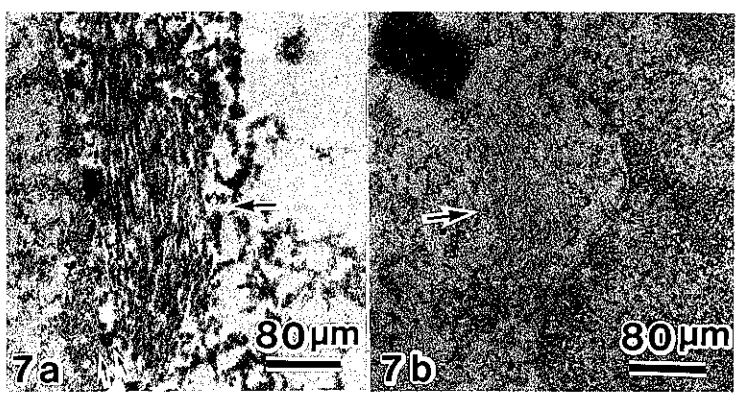


Fig. 4. Immuno-electron microscopic sections (×10,800). ABC-PO method. Ribosomes (arrow) in K562/ADM cells are clearly stained positively with HOT104 Mab (a). The immuno-electron microscopic section in (b) was reacted with non-immune mouse IgGs instead of HOT104 Mab.

Fig. 5. Immuno-electron microscopic sections (×10,800). ABC-PO method. Rough ER in K 562/ADM cells (arrow) is stained intensely with HOT104 Mab (a). The section in (b) was reacted with non-immune mouse IgGs.

Fig. 6. Immuno-electron microscopic sections (×10,800). ABC-PO method. Mitochondria in K562/ADM cells (arrow) are stained strongly with HOT104 Mab (a). The section in (b) was reacted with non-immune mouse IgGs.

Fig. 7. Immuno-electron microscopic sections (×14,000). ABC-PO method. Microfilaments in K562/ADM cells (arrow) and perinuclear membranes (double arrow) are stained positively with HOT104 Mab (a). The section in (b) was reacted with non-immune mouse IgGs.

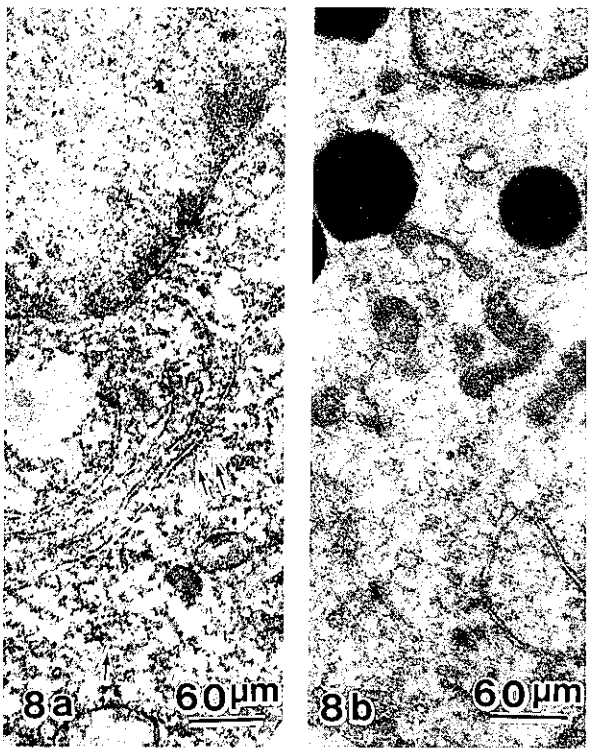


Fig. 8. Immuno-electron microscopic sections (×10,800). ABC-PO method. Rough ER (arrow) and free ribosomes (double arrow) in K562 cells are stained more weakly, compared with those in K562/ADM cells (Figs. 4a and 5a) (a). The section in (b) was reacted with non-immune mouse IgGs.

DISCUSSION

Sorcin, a 22kD calcium-binding protein, is present in the cytoplasm of adriamycin-resistant human myelogenous leukemia cells (K562/ADM) and is localized in free ribosomes, rough ER, mitochondria, microfilaments, and perinuclear membranes. Sorcin was more abundant in K562/ADM than in parental K562 cells. Sorcin was also overproduced in another adriamycin-resistant line derived from A2780, an ovarian cancer line (kindly provided by Drs. Ozols and Hamilton) (data not shown). The amount of sorcin was not in direct proportion to the degree of resistance in the two lines examined, although it is possible that sorcin plays a role in the multidrug resistant mechanism.[8] As sorcin is not a binding protein of adriamycin or vincristine (date of H. Hamada), it may be involved in signal transduction of some sort in multidrug resistance. Further studies will be required to elucidate this point.

Sorcin is one of the calcium-binding proteins, having structural homology with calmodulin and homology with the calcium-binding domains of calpain.[7] If calmodulin and calpain I, as calcium-binding proteins, are located in specific organelles, then other calcium-binding proteins, such as sorcin, might also be specifically localized. Calmodulin is located in ribosomes, rough ER, and smooth ER in Purkinje cells[17] and in the nucleus, cell membrane, and glycogen granules of rat liver cells.[18] Furthermore, calmodulin activity is associated with tubulin dimer.[19] Calmodulin in the secretory ameloblasts of rat incisor is located in the nuclei, mitochondria, cytosol and plasma membranes.[20] Calpain I (a cysteine proteinase activated by micromolar concentrations of Ca^{2+}) has a predominant intracellular location in the I-band region of the extensor digitorum longus muscle of the rat.[21]

On the other hand, sorcin seems to be located in free ribosomes, rough ER, mitochondria, perinuclear membranes and microfilaments. However, there was an inherent technical difficulty in this study. We used 0.5% saponin so that HOT104 Mab would easily enter the cytoplasm. This treatment affected preservation of the cellular organelles and caused diffusion of the immunoreaction products. The presence of sorcin in free ribosomes and rough ER may indicate that sorcin is being synthesized. However, sorcin in mitochondria, perinuclear membranes and microfilaments seems to have some physiological role like those of calmodulin and calpain, besides its yet unknown function in multidrug resistance, e.g., respiratory metabolism, nuclear membrane motility, cell motility, calcium transport, and so forth.

Sorcin is not present in the plasma membrane of K562/ADM cells and does not bind to P-glycoprotein in those cells on the basis of antibody binding experiments with biotinylated MRK 16 Mab. Binding of MRK 16 to K562/ADM cell membranes was not blocked by HOT 104 Mab. Furthermore, Hamada *et al.* have found that the ATPase activity of the P-glycoprotein is not affected by the Mr 22,000 protein (sorcin).[8] The relationship between sorcin and P-glycoprotein in multidrug resistance remains to be solved. It is likely that overproduction of sorcin in K562/ADM cells is a consequence of amplification of P-glycoprotein genes, as is the case in other cells reported in the literature.[7,22,23] The genes encoding sorcin and P-glycoprotein are closely linked.[24]

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