Reversal of P-glycoprotein-mediated multidrug resistance by diallyl sulfide in K562 leukemic cells and in mouse liver

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Multidrug resistance (MDR) mediated by the overexpression of drug efflux protein P-glycoprotein (P-gp) is one of the major obstacles to successful cancer chemotherapy. P-gp acts as an energy-dependent drug efflux pump, reducing the intracellular concentration of structurally unrelated drugs. Modulators of P-gp function can restore the sensitivity of multidrug-resistant cells to such drugs. In the present study, we evaluated the P-gp modulatory potential of diallyl sulfide (DAS), a volatile organosulfur compound present in garlic, known to possess many medicinal properties, including anti-angiogenic and anticarcinogenic activities. For in vitro studies, K562 leukemic cells were made resistant (K562/R) to the cytotoxicity of vinblastine (VBL) by progressive adaptation of the sensitive K562 parental cells to VBL. Cross-resistance of K562/R was found between vincristine (VCR), doxorubicin and other antineoplastic agents. A nontoxic concentration of DAS (8.75 × 10⁻³ M) enhanced the cytotoxic effects of VBL and another vinca alkaloid, VCR, time dependently in VBL-resistant human leukemia (K562/R10) cells but had no effect on the parent (K562/S) cells. The results show that DAS decreased the induced levels of P-gp in resistant cells back to the normal levels as analyzed both qualitatively and quantitatively by western blotting and immunocytochemistry. Furthermore, in vivo combination studies showed that DAS effectively inhibited vinca alkaloid-induced P-gp overexpression in mouse hepatocytes. Quantitation of immunostained tissue sections with image analysis showed that the reduction in P-gp levels was up to 73% for VBL- and 65% for VCR-induced drug resistance. The above features thus indicate that DAS can serve as a novel, non-toxic modulator of MDR and can be used as a dietary adjuvant.

Introduction

One perplexing problem to successful chemotherapy is the development of resistance by tumor cells to multiple chemotherapeutic agents, a phenomenon termed multidrug resistance (MDR). Although the etiology of MDR is multifactorial, overexpression of P-glycoprotein (P-gp), a plasma membrane glycoprotein, remains the most common alteration underlying MDR in laboratory models (1). The expression of P-gp has been linked to the development of MDR in human cancer, particularly in leukemias, lymphomas, multiple myeloma, neuroblastoma and soft tissue sarcoma (2-4). P-gp is a 170-180 kDa protein product of the mdr-1 gene belonging to a superfamily of ATP-binding cassette transporters that actively extrudes a wide range of structurally diverse amphipathic drugs used to treat cancer, including anthracyclins, vinca alkaloids, epipodophyllotoxins and taxanes (5,6). Enhanced efflux of these compounds reduces their intracellular accumulation, with a concomitant decrease in their cytotoxicity.

A plethora of agents have been developed that modify, modulate or reverse the P-gp-mediated MDR phenotype. Many natural and synthetic products of various structures, including calcium channel blockers (verapamil and nifedipine), calmodulin inhibitors (phenothiazines and trifluoperazine), indole alkaloids (reserpine), various steroids (progesterone and tamoxifen), immunosuppressive drugs (cyclosporin and rapamycin) and antibiotics (rifapicin and the tetracyclins) have been shown to modulate P-gp-mediated MDR (2,3,7,8). Anti-Pgp antibodies such as UIC2 (9) and antisense oligodeoxynucleotides directed against mdr-1 gene expression (10) have also been used as MDR reversing agents. Unfortunately, most of these compounds are not useful in tackling the problem of drug resistance at a clinically sustainable level. In some cases there is a lack of potency or, alternatively, the MDR-reversing agent may expose the patient to unacceptable side-effects or toxicity at the doses required for effectiveness (11). These limitations have spurred efforts to search for new compounds with low toxicity and high efficacy in modulation of MDR, such as dexverapamil (12) and cyclosporin derivatives (3,13).

Diallyl sulfide (DAS) is a volatile organosulfur compound (Figure 1) derived from garlic (Allium sativum). DAS is known to possess versatile medicinal properties, including anti-hypotensive, anti-angiogenic, anticarcinogenic and anti-hepatotoxic effects (14-16). Much attention has focused on investigations of the role of DAS in the prevention of cancer (14,17,18). Many reports have shown that DAS has antitumor efficacy in cultured carcinoma cell lines and tumor-bearing mice (19-21). Earlier work from our laboratory has shown the antitumorigenic potential of DAS in transplantable as well as solid mouse skin tumors (22-24). Besides, DAS has also been

Abbreviations: DAS, diallyl sulfide; DMBA, 7,12-dimethyl benzanthracene; DMSO, dimethyl sulfoxide; DXR, doxorubicin; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazole-2-yl)2-diphenyl tetrazolium bromide; P-gp, P-glycoprotein; PBS, phosphate-buffered saline; VPL, verapamil; VBL, vinblastine; VCR, vincristine.

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shown to apoptose induction in 7,12-dimethyl benzanthracene (DMBA)-induced mouse skin tumors (25). It has also been shown to inhibit aflatoxin B1- and N-nitrosodimethylamine-induced liver preneoplastic foci in rats (26). However, so far no report is available on \textit{in vitro} or \textit{in vivo} MDR modulatory activity of DAS.

Because the severe side-effects of the known MDR-reversing agents greatly limit their clinical application, non-toxic DAS may be a better candidate in this regard and is worthy of more intensive studies for its MDR-modulatory effects. In the present study we evaluated the ability of DAS to potentiate the cytotoxicity of vinca alkaloids \textit{in vitro} using vinblastine (VBL)-resistant K562 human leukemic cell lines and \textit{in vivo} using short-term bioassay.

**Materials and methods**

**Cell culture**

The human myelogenous leukemia cell line K562 (27) was procured from the National Center for Cell Sciences (Pune, India). Cells were grown in suspension in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 2 $\times$ 10^{-4} M L-glutamine (Gibco BRL, Gaithersburg, MD) and housed in an incubator at 37$^\circ$C, 99% humidity and 5% CO$_2$. Cells were passaged every other day, checked routinely and found to be free of contamination by mycoplasmas or fungi.

**Drugs**

DAS was purchased from Sigma Chemical Co. (St Louis, MO). Vinblastine sulfate (cytotubulin) (VBL) and vincristine sulfate (cytocristine) (VCR) were obtained from Cipla Ltd, India. Verapamil (VPL), doxorubicin (DXR) and other cytotoxic drugs were procured from Sigma Chemical Co. Stock solutions (10 $\times$ 10$^{-3}$ M) of all drugs were prepared in dimethyl sulfoxide (DMSO) and diluted in culture medium to the required concentrations with the final stock solution (20 $\times$ 10$^{-5}$ M) of all drugs were prepared in dimethyl sulfoxide (DMSO) and stored as aliquots at $\sim$20$^\circ$C. For cell treatments, drugs were further diluted in culture medium to the required concentrations with the final DMSO concentration being <0.5%. DAS was dissolved in ethanol and the stock solution (20 $\times$ 10$^{-2}$ M) was prepared in culture medium.

**Animals**

Adult male Swiss albino mice weighing 20–25 g were obtained from the ITRC animal breeding colony. Animals were kept for a quarantine period of 1 week at a temperature of 25 ± 2$^\circ$C, relative humidity of 55 ± 2% and with photocycle of 12 h light and 12 h dark. Water and food pellets (Lipton, India) were provided ad libitum.

**Drug-resistant subline**

A cell line (K562/R) resistant to the cytotoxicity of VBL was established in our laboratory by the progressive adaptation of the sensitive parental cells (K562/S) to increasing concentrations of VBL. Briefly, K562/S cells were grown initially to increasing concentrations of VBL. Briefly, K562/S cells were grown initially to increasing concentrations of VBL. At each concentration, a subculture was repeated using 1- to 5-fold increments in VBL concentration over a span of 1 year until cells tolerated the continuous presence of 10 $\times$ 10$^{-6}$ M VBL. Cells were subcultured at this concentration for >100 population doublings. Cells that were adapted to growth at 0.5, 2.5, 5.0, 7.5 and 10 $\times$ 10$^{-6}$ M VBL during this process were subcultured separately for at least 100 passages, after which any VBL-induced growth inhibition had disappeared. The sublines selected in this way were termed K562/R0.5, K562/R2.5, K562/R5.0, K562/R7.5 and K562/R10, indicating the VBL concentration (10$^{-6}$ M) at which they were able to grow without detectable inhibition. The K562/R10 subline was used further in the study and the MDR phenotype was maintained by continuously growing the cells in the presence of 10 $\times$ 10$^{-6}$ M VBL. VBL was withdrawn from the medium 1 week before using K562/R10 cells for further study.

**Sensitization of resistant cells to anticancer drug toxicity by DAS**

To determine whether DAS can sensitize multidrug-resistant cells to the cytotoxicity of VBL or VCR, K562/S and K562/R (1 × 10$^6$) cells were plated in 96-well microtiter plates in 100 $\mu$l of culture medium. After 24 h of plating, cells were incubated with varying concentrations of DAS (0.1-87.5 $\times$ 10$^{-3}$ M). Cells were then incubated for 24, 48 or 72 h, after which viability was assessed by the reduction of 3-(4,5-dimethylthiazole-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) (28). MTT [10 $\mu$l of a 5 mg/ml stock solution in phosphate-buffered saline (PBS)] was added to each well of the microtiter plate and the plates were incubated for an additional 4 h in the dark at 37$^\circ$C. The medium was then aspirated from the wells and the blue formazon product (MTS) was added to each well of the microtiter plate reader (Bio-Rad, Tokyo, Japan). Each data point was replicated in triplicate. IC$_{50}$ values and inhibition curves connecting the data points were obtained using the non-linear regression program Graph Pad Prism 3.03.

**Cellular accumulation of DAS**

To estimate DXR in \textit{vitro}, 1 $\times$ 10$^5$ cells/ml were resuspended in culture tubes in the presence or absence of different concentrations of DAS. DXR (5 $\times$ 10$^{-6}$ M) was added to the cells, gently mixed and incubated at 37$^\circ$C. Tubes were removed at 45 min and cells were analyzed for intracellular accumulation by a BD-LSR flow cytometer (Becton-Dickinson, San Jose, CA). A total of 10 000 cells were counted. Mean fluorescence was recorded from the histogram and the data are expressed in the text as mean fluorescence channel numbers.

**Cell count and preparation of cell extracts for immunoblotting**

About 5 $\times$ 10$^5$ cells/ml were grown in T25 cm$^2$ flasks as suspensions with and without DAS (8.75 $\times$ 10$^{-3}$ M) and harvested after 24, 48 or 72 h exposure. Viable cells were counted by trypan blue exclusion assay. The cells were further centrifuged at 1000 r.p.m. for 10 min, washed once in PBS and then resuspended in prewarmed sample buffer (0.5 M Tris-HCl, pH 6.8 containing 10% w/v SDS, 10% v/v glycerol, 5% v/v β-mercaptoethanol and 0.1% w/v bromophenol blue). Cells were boiled in sample buffer for 10 min, cooled, sonicated using a VibraCell ultrasonicator (Sonics & Materials Inc., Newtown, CT) on ice and stored at $\sim$80$^\circ$C until use.

**Modulation of P-gp-mediated MDR in \textit{vivo}**

To determine the efficacy of DAS in modulating P-gp-mediated drug resistance, a short-term animal bioassay was performed. Mouse hepatocytes were sensitized with VBL (2 mg/kg body wt) or VCR (1 mg/kg body wt) for 3 consecutive days (days 1–3) i.p. in 0.9% saline. DAS (5 mg/kg body wt) was given orally by gastric intubation on day 3, 1 h after the drug treatment. In order to check the efficacy level of DAS, VPL, a known MDR modulator, was also included in the study at a dose of 10 mg/kg body wt as per the schedule for DAS. The treatment schedules for different groups (comprising six animals each) are summarized in Table I.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>Group I (untreated)</td>
<td>No treatment</td>
</tr>
<tr>
<td>Group II (DAS)</td>
<td>DAS was given orally at a dose of 5 mg/kg body wt in 0.9% saline on day 3</td>
</tr>
<tr>
<td>Group III (VBL)</td>
<td>VBL was given at a dose of 10 mg/kg body wt in 0.9% saline by i.p. administration on day 3</td>
</tr>
<tr>
<td>Group IV (VBL + DAS)</td>
<td>VBL at a dose of 2 mg/kg body wt by i.p. administration for 3 consecutive days in 0.9% saline</td>
</tr>
<tr>
<td>Group V (VBL + DAS)</td>
<td>VBL at a dose of 2 mg/kg body wt by i.p. administration for 3 consecutive days. DAS was given orally at a dose of 5 mg/kg body wt in 0.9% saline 1 h after the last dose of VBL</td>
</tr>
<tr>
<td>Group VI (VBL + VPL)</td>
<td>VBL was given as in group V. VPL was given at a dose of 10 mg/kg body wt by i.p. administration in 0.9% saline 1 h after the last dose of VBL</td>
</tr>
<tr>
<td>Group VII (VCR)</td>
<td>VCR at a dose of 1 mg/kg body wt by i.p. administration for 3 consecutive days in 0.9% saline</td>
</tr>
<tr>
<td>Group VIII (VCR + DAS)</td>
<td>VCR as in group VII. DAS was given orally at a dose of 5 mg/kg body wt in 0.9% saline 1 h after the last dose of VCR</td>
</tr>
<tr>
<td>Group IX (VCR + VPL)</td>
<td>VCR at a dose of 1 mg/kg body wt by i.p. administration for 3 consecutive days. VPL was given at a dose of 10 mg/kg body wt by i.p. administration in 0.9% saline 1 h after the last dose of VCR</td>
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All the animals were killed by cervical dislocation on day 4, i.e. 24 h after drug treatment. Liver specimens were excised, rinsed with prechilled physiological buffered saline, immediately frozen in liquid nitrogen and stored at $-80^\circ$C until use.

**Immunoblotting**

Immunoblots were carried out as described by Towbin et al. (29) in plasma membrane enriched fractions from liver specimens as well as whole cell lysates of K562/S and K562/R cells. Protein concentration was estimated by the routine method of Lowry et al. (30) using BSA as a standard. Proteins (50–150 µg) were resolved on 7.5% SDS-polyacrylamide gels (31) and electroblotted onto nitrocellulose membranes. The blots were blocked overnight with 3% non-fat dried milk and probed with anti-P-gp monoclonal antibody (0.5 µg/ml) clone JSB1 (Boehringer Mannheim, Mannheim, Germany). Immunoblots were detected with horseradish peroxidase-conjugated secondary antibody with the chromagen 3,3'$\text{o}$-diaminobenzidine tetrahydrochloride.

**Immunocytochemical/immunohistochemical staining**

For immunocytochemistry, cell smears of K562/S and K562/R cells (1 x 10$^6$/ml) were prepared using Autosmear (CF120; Sakura, Tokyo, Japan) at 1000 r.p.m. for 10 min. For immunohistochemistry cryostat sections (10 µm) of frozen liver tissues were cut and washed with cold physiological buffered saline. Sections were incubated with mouse monoclonal antibody JSB1 (Boehringer Mannheim, Mannheim, Germany), visualized using horseradish peroxidase-conjugated secondary antibody and the chromagen 3,3'$\text{o}$-diaminobenzidine tetrahydrochloride.

![Fig. 2. (a) Cell viability among different K562/VBL sublines. Resistant variants were incubated for varying time intervals in the presence of 10 x 10$^{-6}$ M VBL and cytotoxicity was determined by the MTT assay as described in Materials and methods. Regression analysis was performed to determine the correlation between the cytotoxicity of VBL and the degree of resistance. The correlation coefficient was r > 0.9 (P < 0.001). (b) Immunoblot analysis of P-gp overexpression in resistant (K562/R10) cells over parental sensitive (K562/S) cells. Total cellular protein (100 µg) from K562/R10 (lane a), K562/R5 (lane b), K562/R2.5 (lane c) and K562/S (lane d) cells was resolved by 7.5% SDS-PAGE after electrotransfer onto nitrocellulose membrane, reacted with anti-P-gp monoclonal antibody (clone JSB1) and visualized using horseradish peroxidase-linked secondary antibody with the chromagen 3,3'$\text{o}$-diaminobenzidine tetrahydrochloride.](https://academic.oup.com/carcin/article-abstract/25/6/941/2390679)

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$_{50}$ (10$^{-6}$ M) K562</th>
<th>IC$_{50}$ (10$^{-6}$ M) K562/R10</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleomycin</td>
<td>3.25</td>
<td>9.10</td>
<td>2.8</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.014</td>
<td>0.032</td>
<td>2.3</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.042</td>
<td>3.86</td>
<td>92</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.03</td>
<td>0.046</td>
<td>1.5</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.07</td>
<td>14.86</td>
<td>212</td>
</tr>
<tr>
<td>Vinristine</td>
<td>0.12</td>
<td>17.27</td>
<td>144</td>
</tr>
</tbody>
</table>

The cross resistance of K562/R10 to a variety of drugs with widely disparate mechanisms of cytotoxic action was investigated. Cells were grown in suspension culture in the continuous presence of the drug for 72 h. The IC$_{50}$ was determined by plotting the logarithm of the drug concentration against the number of dead cells. SD was usually within 10% of the mean. Fold resistance was determined by dividing the IC$_{50}$ for the resistant line K562/R10 by the IC$_{50}$ for the sensitive K562/S cells.
liver tissues were cut, fixed in 4% formal saline and endogenous peroxidase activity was quenched with a solution of H2O2 in methanol (333 μl in 100 ml). After quenching, non-specific binding was minimized by incubation with normal goat serum (1:30) for 2 h at 4°C. The slides were sequentially incubated in a moist chamber with anti P-gp antibody (0.25 μg/ml) for 72 h. After the incubation period, the smears/sections were again incubated for 2 h with normal goat serum and then with horseradish peroxidase-conjugated anti-goat IgG for 6 h at 4°C. The slides were washed with PBS between incubations and, after a final rinse with PBS, finally washed with 0.1 M acetate buffer (pH 5.2). The color was developed using the chromagen 3,3'-diaminobenzidine tetrahydrochloride.

Image analysis
The immunostained slides for P-gp were analyzed under a microscope (Leica) with an attached CCD camera (JVC). Quantitative stereology was performed with Leica QWin 500 Image analysis software for each slide in triplicate, with at least six microscopic fields. The statistical analyses were done using Student’s t-test and P < 0.05 was considered significant.

Results
Resistance characteristics of K562/VBL
Exposure of K562/S cells to 10 × 10⁻⁶ M VBL resulted in 75% cell death after 72 h. The viabilities of resistant K562 variants were of higher magnitudes and maximum survival (91-96%) was observed for the K562/R7.5 and K562/R10 sublines, respectively, at the same concentration of VBL (Figure 2a). A significant correlation (correlation coefficient r > 0.9, P < 0.001) was observed between the cells survival rate and the degree of resistance in various strains resistant to the cytotoxicity of 10 × 10⁻⁶ M VBL (Figure 2a). The data for determining the VBL sensitivity of the parent K562 cells and the VBL-resistant subline K562/R10 are shown in Table II. The IC₅₀ for the parental cells is 0.07 × 10⁻⁶ M and for K562/R10 cells is 14.86 × 10⁻⁶ M. The ratio of these values is defined as the relative resistance, which is 212.3 for K562/R10 cells (Table II). Similarly, K562/R10 cells were found to be highly cross-resistant to VCR (144-fold), DXR (92-fold) compared with K562/S cells, but only slightly resistant to bleomycin (2.8-fold), cisplatin (2.3-fold) and methotrexate (1.53-fold) (Table II).

Since the amount of P-gp is correlated with the degree of resistance, particularly at low to moderate levels of resistance (6), the levels of P-gp among these variants were also characterized on western blots using anti-P-gp monoclonal antibody clone JSB-1. K562/R10 cells were found to overexpress P-gp in comparison with the parental sensitive cells (Figure 2b).

Sensitization of resistant cells by DAS
The ability of DAS to sensitize resistant K562/R10 cells to the cytotoxicity of VBL was evaluated. DAS was assessed at
increasing concentrations for its ability to sensitize K562/R10 cells to the cytotoxicity of VBL. These concentrations of VBL represented the maximum concentration that had no effect on K562/R10 cell viability. The results revealed that DAS inhibited the growth of the resistant cell line to a much higher extent than the parental sensitive cell line. The results showed that DAS sensitized K562/R10 cells to the cytotoxicity of VBL with 50% sensitization at 72 h exposure to a concentration of 8.75 \times 10^{-3} \text{ M}, which had no effect on the viability of K562/R10 and K562/S cells (Figure 3). At concentrations higher than 8.75 \times 10^{-3} \text{ M} DAS becomes cytotoxic to both resistant and sensitive K562 cells after 48 and 72 h. However, at 24 h

![Fig. 5. Photomicrographs showing immunostaining of K562/S and K562/R10 cells. (a) K562/S cells; (b) K562/R10 cells; (c) K562/R10 cells exposed to DAS for 48 h; (d) K562/R10 cells exposed to DAS for 72 h.](image)

![Table III. Quantitation of P-glycoprotein expression in the multidrug-resistant cell line K562](table)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Area (%)</th>
<th>Area (mm²) (mean ± SE)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562/S</td>
<td>0.64</td>
<td>4.21 ± 0.54</td>
<td>23</td>
</tr>
<tr>
<td>K562/R10</td>
<td>6.75</td>
<td>24.25 ± 3.16</td>
<td>54*</td>
</tr>
<tr>
<td>K562/R10 + DAS (24)</td>
<td>5.47</td>
<td>18.65 ± 2.25</td>
<td>79*</td>
</tr>
<tr>
<td>K562/R10 + DAS (48)</td>
<td>2.83</td>
<td>11.16 ± 1.68</td>
<td></td>
</tr>
<tr>
<td>K562/R10 + DAS (72)</td>
<td>1.04</td>
<td>5.08 ± 0.84</td>
<td></td>
</tr>
<tr>
<td>K562/S + DAS (72)</td>
<td>0.84</td>
<td>4.54 ± 0.63</td>
<td></td>
</tr>
</tbody>
</table>

*Values are significantly different (P < 0.001) over K562/R10 (group II).
On the basis of the results of these investigations, we reported the modulatory effects of DAS on vinca alkaloid-induced, P-gp-mediated MDR in K562 cancer cells. DAS is a known MDR-reversing agent, the VBL-induced levels of P-gp were found to be comparable to that of normal levels. Similarly, VCR-induced levels of P-gp were reduced to normal levels with the administration of DAS and VPL (Figure 7b). Exposure to either DAS or VPL alone had no effect on the P-gp expression (Figure 7a and b).

**Immunohistochemistry**

Analyses of immunostained cryostat liver sections using the Leica QWin Image analysis system showed elevated levels of P-gp following vinca alkaloid exposure which was reversed back to the normal level with DAS (Figure 8). Areas of $-53.19 \pm 3.2$ and $-47.95 \pm 4.3 \text{ mm}^2$ were shown to be positive for P-gp with VBL and VCR treatment, respectively, compared with $9.43 \pm 0.7 \text{ mm}^2$ for the control, per measured frame of 202.5 mm$^2$ (Table IV). Co-administration of VPL reduced the area of expression of P-gp from 26 to 8% in the case of VBL and from 24 to 9% in the case of VCR (Table IV). Similarly, DAS exposure resulted in a decrease in vinca alkaloid-induced P-gp levels to 7–9%, which was comparable with that of the control (Table IV). Therefore, ~65–73% inhibition of vinca alkaloid-induced P-gp expression was shown by DAS in comparison with 65–70% inhibition by VPL (Table IV).

**Discussion**

Cross-resistance to multiple classes of chemotherapeutic agents is a major problem in the treatment of several types of human cancers (32,33). A major mechanism of this resistance is the enhanced efflux of a wide variety of structurally distinct classes of chemotherapeutic agents due to the overexpression of P-gp (34). Resistance to vinca alkaloids has mainly been associated with P-gp-mediated MDR, while several mammalian cell lines selected for VBL or VCR resistance have displayed lower accumulation of these agents related to P-gp or mdr gene overexpression (35). Several MDR-reversing agents have been identified and several others are in various stages of clinical development (11,36,37).

On the basis of the results of these investigations, we reported the modulatory effects of DAS on vinca alkaloid-induced, P-gp-mediated MDR in K562 cancer cells. DAS is a

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**Fig. 6.** Effect of DAS on doxorubicin accumulation in K562/S and K562/R10 cells. $1 \times 10^6$ cells were loaded with doxorubicin ($5 \times 10^{-6} \text{ M}$) in the presence or absence of different concentrations of DAS for 45 min. The drug uptake was measured using flow cytometry (BD-LSR) in K562/S (open circles) and K562/R10 (solid circles) cells. The dotted line (open triangles) shows K562/R10 cells in the absence of DAS. Values are expressed as means $\pm$ SD fluorescence numbers. *$P < 0.05$.**
well-known antimutagenic and anticarcinogenic agent of natural origin (14–17). Several compounds of dietary origin, such as indole-3-carbinol (38,39) and tea polyphenols (40,41), have already been shown in our laboratory and elsewhere to be effective in modulating P-gp-mediated MDR in both in vitro and in vivo assays.

The results of the present study clearly demonstrate that DAS is a novel, selective and highly potent modulator of P-gp-mediated MDR in human K562 leukemic cells and in rodent liver. The in vitro P-gp modulating potency has been demonstrated by the potentiation of cytotoxic drug activity using the MTT assay. In the cytotoxicity assay a complete reversal of the VBL resistance of human leukemia cell line K562 was achieved in the presence of DAS in a time-dependent manner (Figure 3). In contrast to the modulatory activity in the resistant cell line, DAS has no apparent effect on cytotoxic drug activity in the P-gp non-expressing parental (susceptible) cell line (K562/S). DAS also increased the accumulation of DXR in resistant cells (K562/R10), to an extent almost comparable with that observed in parental K562 cells incubated without DAS (Figure 6). Furthermore, overexpression of P-gp in K562R/10 cells was effectively inhibited by DAS, as is evident from the immunoblotting assays (Figure 4). Quantitation of immunocytochemically stained K562 cells showed that this inhibition of P-gp expression was time dependent (Figure 5 and Table III). Therefore, these results emphatically show for the first time that DAS possesses inhibitory activity against the growth of K562/R10 human leukemic cells but not the parental K562/S cell line. The promising activity of DAS demonstrated in vitro was further confirmed in in vivo efficacy studies in mouse liver. Sensitization of mouse liver with VBL or VCR for 3 consecutive days resulted in enhanced expression of P-gp, which was found to be reversed to near normal levels by DAS administration (Figure 7a and b). This reversal in P-gp expression was up to 73% for VBL- and 65% for VCR-induced MDR (Figure 8 and Table IV).

The mechanism of action of the MDR-reversing potential of DAS appears to be mediated through the modulation of P-gp expression. K562/VLB and K562/DXR cells are known to overexpress P-gp, but K562/S cells do not (42). The ability of DAS to increase the accumulation of DXR in K562/R10 cells supports the conclusion that DAS acts by interfering with a process associated with the expression of P-gp. However, other possibilities cannot be ruled out at the moment and further studies are required for a complete understanding of the underlying mechanism of DAS-mediated MDR reversal. Besides, most MDR-reversing compounds share some common characteristics, such as cyclicity, lipophilicity and a positive or neutral charge at physiological pH (43). The MDR-reversing properties of DAS might also be attributed to its lipophilic nature and electrophilic sulfur atom. Furthermore, DAS undergoes extensive oxidation at various positions (the sulfur atom, the allylic carbon and the terminal double bonds) in the molecule. Cytochrome P450 enzyme-mediated oxidation at the sulfur atom of DAS produces diallyl sulfoxide and diallyl sulfone (44). These metabolites are further converted to epoxide intermediates (45) thought to be responsible for the biological effects of DAS.

Thus, from the present study we can conclude that the remarkably low levels of toxicity of DAS coupled with its MDR-reversing capability in K562 leukemic cells and mouse liver may contribute to the known anticarcinogenic properties of this compound. An advantage of using newly identified dietary agents as modulators of MDR is that they enhance antitumor activity and exhibit little or virtually no side-effects without any further increase in the medication burden on the
The use of natural agents mainly of dietary origin, such as DAS, early in the disease process may retard or prevent the appearance of resistant neoplastic clones. Therefore, identification of newer agents with acceptable, little or no drug-drug interactions and with chemotherapeutic activity is the need of the hour, as observed in the present study with DAS.

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References


Table IV. Quantitation of P-gp expression in short term in vivo bioassay

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Area (%)</th>
<th>Area (mm²) (mean ± SE)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>6</td>
<td>9.43 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>DAS</td>
<td>7</td>
<td>17.16 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>VPL</td>
<td>8</td>
<td>13.41 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>VBL</td>
<td>26</td>
<td>53.19 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>VBL + DAS</td>
<td>7</td>
<td>14.39 ± 0.7</td>
<td>73a</td>
</tr>
<tr>
<td>VI</td>
<td>VBL + VPL</td>
<td>8</td>
<td>15.26 ± 0.9</td>
<td>71a</td>
</tr>
<tr>
<td>VII</td>
<td>VCR</td>
<td>24</td>
<td>47.95 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>VCR + DAS</td>
<td>9</td>
<td>17.88 ± 0.8</td>
<td>65b</td>
</tr>
<tr>
<td>IX</td>
<td>VCR + VPL</td>
<td>9</td>
<td>17.33 ± 1.3</td>
<td>64b</td>
</tr>
</tbody>
</table>

*aInhibition (%) was calculated over group IV.
*bInhibition (%) was calculated over group VII.

Fig. 8. Immunohistochemical staining of P-gp expression in mouse liver. (a) Untreated; (b) DAS; (c) VBL; (d) VBL + DAS; (e) VCR; (f) VCR + DAS.


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