Carcinogenesis vol.25 no.6 pp.941–949, 2004
DOI: 10.1093/carcin/bgh060

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

Annu Arora, Kavita Seth and Yogeshwer Shukla[1]

Environmental Carcinogenesis Division, Industrial Toxicology Research Centre, PO Box 80, M.G.Marg, Lucknow-226001, India

[1]To whom correspondence should be addressed. Fax: +91 522 2228227; Email: yogeshwer_shukla@hotmail.com

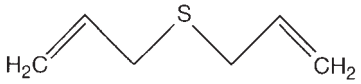
Multidrug resistance (MDR) mediated by the overexpression of drug efflux protein P-glycoprotein (P-gp) is one of the major obstacles to successful cancer chemotherapy. P-gp acts as an energy-dependent drug efflux pump, reducing the intracellular concentration of structurally unrelated drugs. Modulators 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 antimutagenic 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 non-toxic 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 trifuroperazine), 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, such as anti-hypotensive, antimutagenic, 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



Mol. formula $[(CH_2{=}CHCH_2)_2S]$
Mol. Weight 114.20
CAS R. No. 592-88-1

Fig. 1. Structure of diallyl sulfide.

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.

shown to induce apoptosis in 7,12-dimethyl benzanthracene (DMBA)-induced mouse skin tumors (25). It has also been shown to inhibit aflatoxin B_1- and *N*-nitrosodiethylamine-induced liver preneoplastic foci in rats (26). However, so far no report is available on *in vitro* or *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 *in vitro* using vinblastine (VBL)-resistant K562 human leukemic cell lines and *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 2×10^{-3} M L-glutamine (Gibco BRL, Gaithersburg, MD) and housed in an incubator at 37°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 (cytoblastine) (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 (10×10^{-5} M) of all drugs were prepared in dimethyl sulfoxide (DMSO) and were stored as aliquots at −20°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 (20×10^{-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°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 in the continuous presence of 0.5×10^{-6} M VBL (K562/R0.5) until the population doubling time was similar to that of control cultures. This procedure was repeated using 1- to 5-fold increments in VBL concentration over a span of 1 year until cells tolerated the continuous presence of 10×10^{-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 10×10^{-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 10×10^{-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 (1×10^5) cells were plated in 96-well microtiter plates in 100 μl of culture medium. After 24 h of plating, cells were incubated with varying concentrations of DAS (0.1–87.5×10^{-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-diphenyl tetrazolium bromide (MTT) (28). MTT [10 μl 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°C. The medium was then aspirated from the wells and the blue formazon product was dissolved in 100 μl of DMSO. The plates were allowed to stand for 10 min, after which they were read at 570 nm using a spectrophotometric 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 DXR

To estimate DXR *in vitro*, 1×10^6 cells/ml were resuspended in culture tubes in the presence or absence of different concentrations of DAS. DXR (5×10^{-6} M) was added to the cells, gently mixed and incubated at 37°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 5×10^5 cells/ml were grown in T25 cm^2 flasks as suspensions with and without DAS (8.75×10^{-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 −80°C until use.

Modulation of P-gp-mediated MDR in 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 I. Treatment schedule for evaluation of *in vivo* P-gp modulatory activity of DAS

Group	Treatment
Group I (untreated)	No treatment
Group II (DAS)	DAS was given orally at a dose of 5 mg/kg body wt in 0.9% saline on day 3
Group III (VPL)	VPL was given at a dose of 10 mg/kg body wt in 0.9% saline by i.p. administration on day 3
Group IV (VBL)	VBL at a dose of 2 mg/kg body wt by i.p. administration for 3 consecutive days in 0.9% saline
Group V (VBL + DAS)	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
Group VI (VBL + VPL)	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
Group VII (VCR)	VCR at a dose of 1 mg/kg body wt by i.p. administration for 3 consecutive days in 0.9% saline
Group VIII (VCR + DAS)	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
Group IX (VCR + VPL)	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

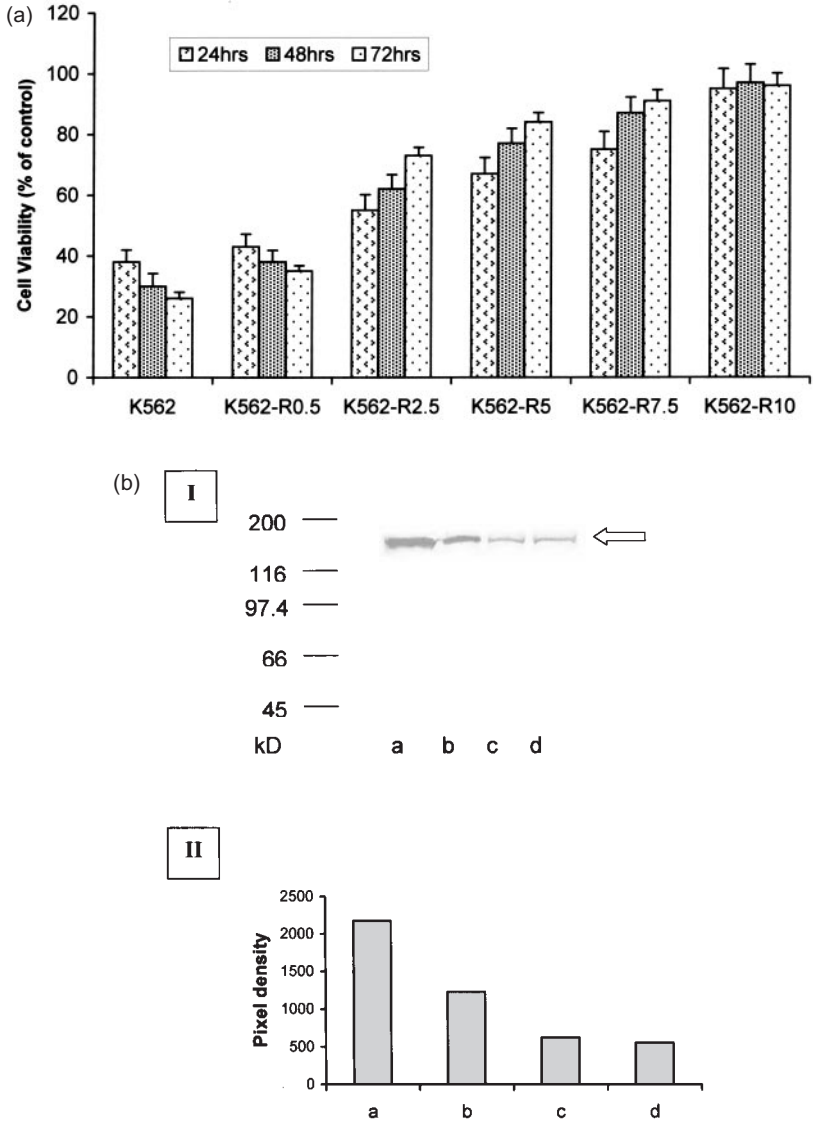


Fig. 2. (**a**) Cell viability among different K562/VBL sublines. Resistant variants were incubated for varying time intervals in the presence of 10×10^{-6} M VBL and cytotoxicity was determined by the MTT assay as described in Materials and methods. Regression analysis was performed to determine the 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′-diaminobenzidine tetrahydrochloride.

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°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 anti-mouse IgG using the chromagen 3,3′-diaminobenzidine tetrahydrochloride.

Immunocytochemical/immunohistochemical staining

For immunocytochemistry, cell smears of K562/S and K562/R cells (1×10^6/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

Table II. Sensitivity of K562 and K562/R10 leukemic cells to cytotoxic agents

Drug	IC_{50} (10^{-6} M)		Relative resistance
	K562	K562/R10	
Bleomycin	3.25	9.10	2.8
Cisplatin	0.014	0.032	2.3
Doxorubicin	0.042	3.86	92
Methotrexate	0.03	0.046	1.5
Vinblastine	0.07	14.86	212
Vincristine	0.12	17.27	144

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.

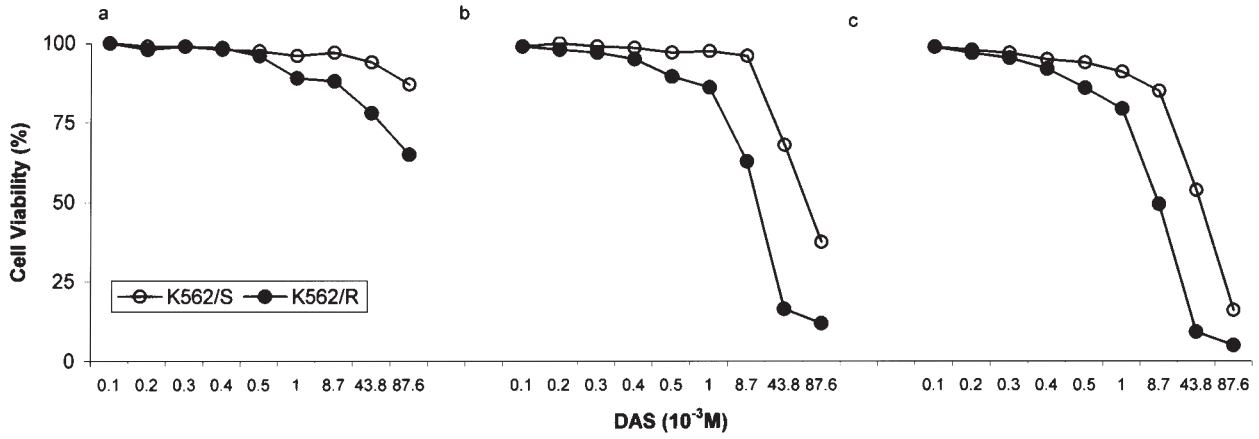


Fig. 3. Cell viability assay of both sensitive (K562/S) and resistant (K562/R10) cells in the presence of increasing concentrations of DAS (0.1–87.5 × 10^{-3} M). Cell viability was assessed after (**a**) 24, (**b**) 48 and (**c**) 72 h by MTT reduction as described in Materials and methods.

liver tissues were cut, fixed in 4% formal saline and endogenous peroxidase activity was quenched with a solution of H_2O_2 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 IC_{50} 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).

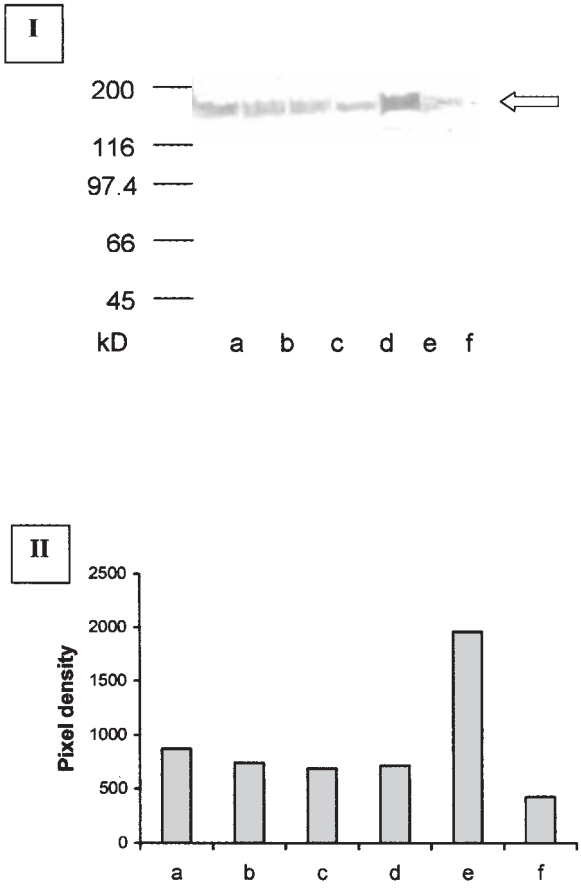


Fig. 4. Immunoblot for P-gp modulation by DAS in K562/R10 cells. Lane a, K562/S cells exposed to DAS for 72 h; lane b, K562/R10 cells exposed to DAS for 72 h; lane c, K562/R10 cells exposed to DAS for 48 h; lane d, K562/R10 cells exposed to DAS for 24 h; lane e, K562/R10 cells; lane f, K562/S cells.

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

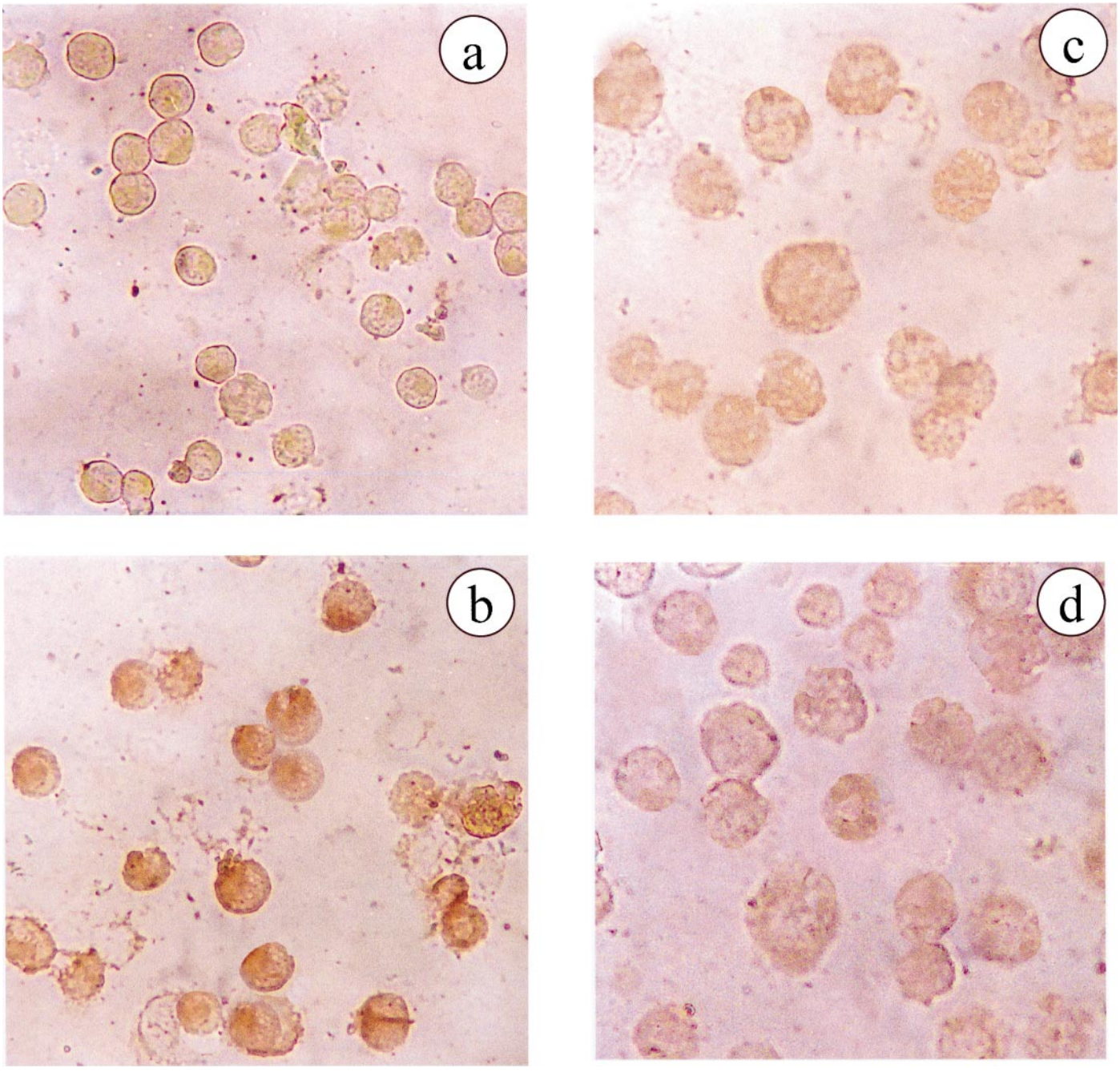


Fig. 5. Photomicrographs showing immunostaining of K562/S and K562/R10 cells. (**a**) K562/S cells; (**b**) K562/R10 cells; (**c**) K562/R10 cells exposed to DAS for 48 h; (**d**) K562/R10 cells exposed to DAS for 72 h.

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 8.75×10^{-3} M, which had no effect on the viability of K562/R10 and K562/S cells (Figure 3). At concentrations higher than 8.75×10^{-3} M DAS becomes cytotoxic to both resistant and sensitive K562 cells after 48 and 72 h. However, at 24 h

Table III. Quantitation of P-glycoprotein expression in the multidrug-resistant cell line K562

Treatment	Area (%)	Area (mm^2) (mean ± SE)	Inhibition (%)
K562/S	0.64	4.21 ± 0.54	
K562/R10	6.75	24.25 ± 3.16	
K562/R10 + DAS (24)	5.47	18.65 ± 2.25	23
K562/R10 + DAS (48)	2.83	11.16 ± 1.68	54[a]
K562/R10 + DAS (72)	1.04	5.08 ± 0.84	79[a]
K562/S + DAS (72)	0.84	4.54 ± 0.63	

[a]Values are significantly different ($P < 0.001$) over K562/R10 (group II).

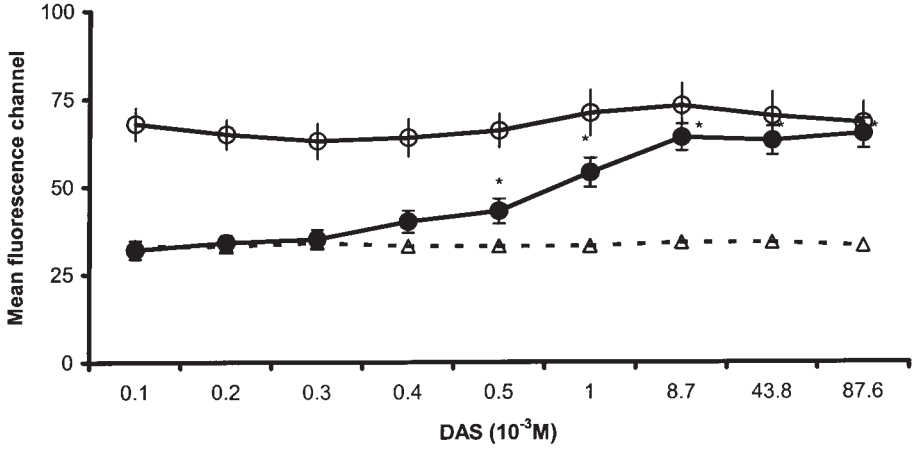


Fig. 6. Effect of DAS on doxorubicin accumulation in K562/S and K562/R10 cells. 1×10^6 cells were loaded with doxorubicin (5×10^{-6} M) in the presence or absence of different concentrations of DAS for 45 min. The drug uptake was measured using flow cytometry (BD-LSR) in K562/S (open circles) and K562/R10 (solid circles) cells. The dotted line (open triangles) shows K562/R10 cells in the absence of DAS. Values are expressed as means ± SD fluorescence numbers. $^*P < 0.05$.

exposure 22% sensitization of K562/R10 cells was observed with 43.78×10^{-3} M DAS (Figure 3). In the case of parental K562/S cells, 50% sensitization was observed at 56.8×10^{-3} M DAS. Therefore, the fold reversal caused by DAS in K562/R10 cells was found to be 6.5.

Furthermore, to clarify that this differential growth inhibitory activity of DAS on resistant and parental cells was due to the modulation of P-gp expression, a specific monoclonal antibody, JSB-1, which recognizes an internal epitope of P-gp was used for western blotting and immunocytochemistry. The parental K562/S line expresses low but detectable amounts of P-gp whereas resistant K562/R10 cells hyperexpress P-gp (Figures 2b and 4). DAS (8.75×10^{-3} M) exposure resulted in a time-dependent reduction in P-gp levels in K562/R10 cells, with a maximum being observed at 72 h (Figure 4). Quantitation of these cells following immunocytochemical staining showed that this inhibition of P-gp expression was 23, 54 and 79% at 24, 48 and 72 h, respectively (Figure 5 and Table III). DAS had no effect on P-gp expression in parental K562/S cells.

Effect of DAS on DXR uptake

To explore the effect of DAS on intracellular accumulation of DXR, both K562/R10 and K562/S cells were incubated with DXR in the presence or absence of various concentrations of DAS. DXR effectively accumulated in K562/S cells at 37°C and DAS at 8.75×10^{-3} M did not affect this accumulation. In K562/R10 cells the accumulation of DXR was extremely reduced as compared with sensitive K562/S cells at 37°C (Figure 6). DAS restored the DXR accumulation in K562/R10 cells to a level comparable to that of K562/S cells in a dose-dependent manner. Thus, DAS at 8.75×10^{-3} M enhanced the accumulation of DXR in K562/R10 cells to an extent almost comparable with that observed in parental K562 cells incubated without DAS.

Modulation of P-gp by DAS in vivo

A detectable amount of P-gp was observed in normal mouse liver (Figure 7a). Sensitization of liver cells with VBL for 3 consecutive days resulted in elevated levels of P-gp, which was reduced to near the normal level by the administration of DAS (Figure 7a). In the group given animals VPL, a well-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 ± 3.2 and ~47.95 ± 4.3 mm^2 were shown to be positive for P-gp with VBL and VCR treatment, respectively, compared with 9.43 ± 0.7 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

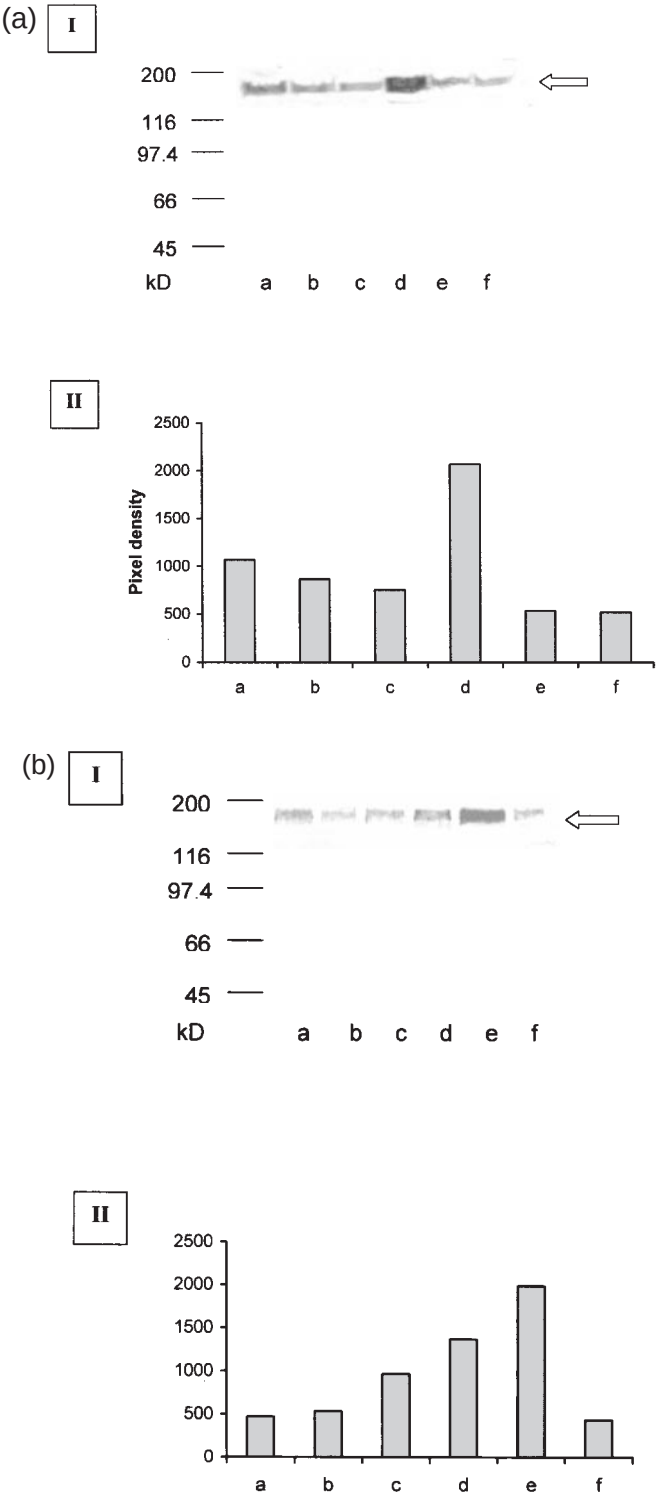


Fig. 7. (**a**) Immunoblot for the *in vivo* reversal of VBL-induced P-gp overexpression by DAS, Lane a, VBL + VPL; lane b, VBL + DAS; lane c, VPL; lane d, VBL; lane e, DAS; lane f, untreated. (**b**) Immunoblot for the *in vivo* reversal of VCR-induced P-gp overexpression by DAS. Lane a, VPL; lane b, VCR + VPL; lane c, DAS; lane d, VCR + DAS; lane e, VCR; lane f, untreated.

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

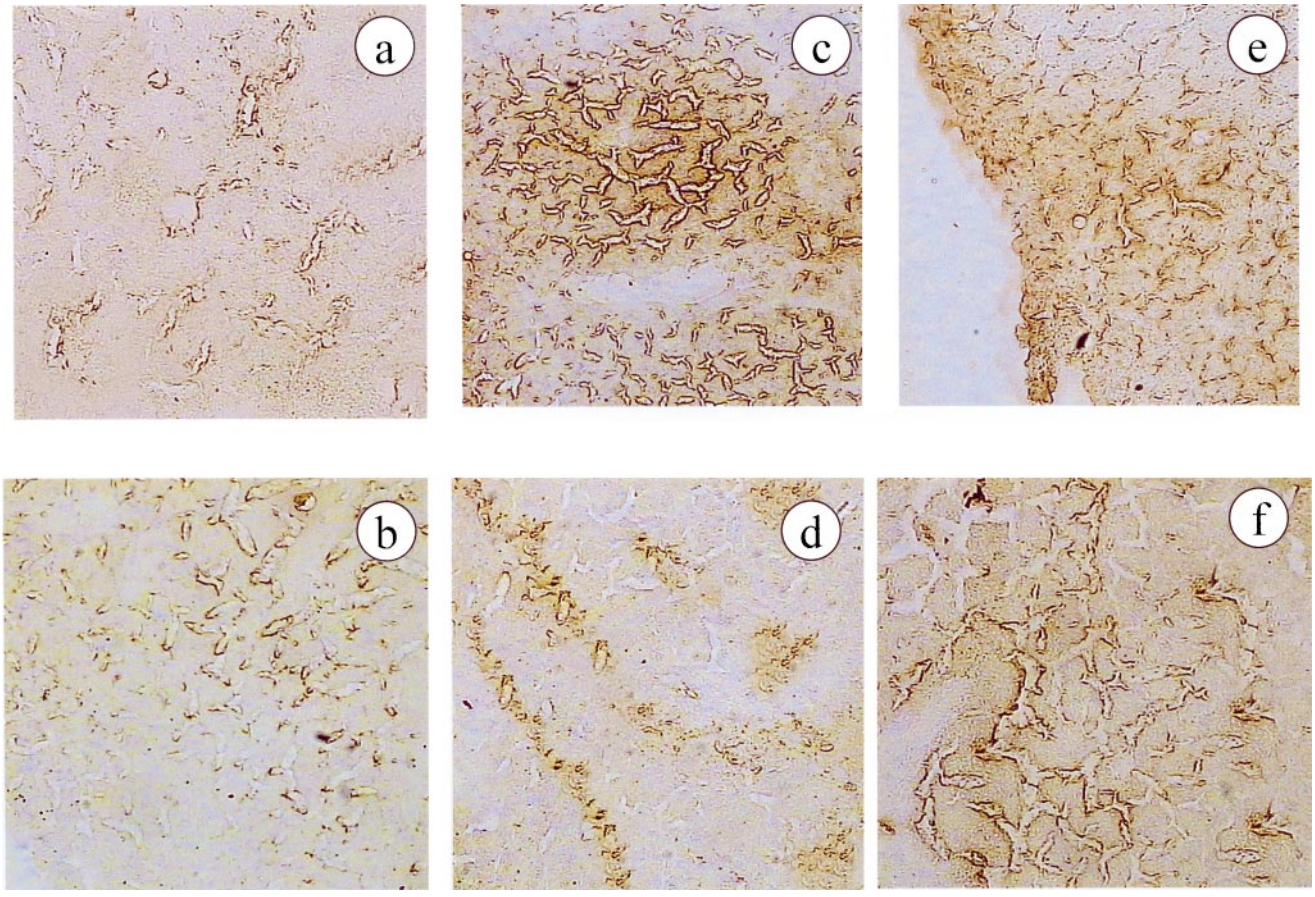


Fig. 8. Immunohistochemical staining of P-gp expression in mouse liver. (**a**) Untreated; (**b**) DAS; (**c**) VBL; (**d**) VBL + DAS; (**e**) VCR; (**f**) VCR + DAS.

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

Group	Treatment	Area (%)	Area (mm^2) (mean ± SE)	Inhibition (%)
I	Control	6	9.43 ± 0.7	
II	DAS	7	17.16 ± 1.4	
III	VPL	8	13.41 ± 1.0	
IV	VBL	26	53.19 ± 3.2	
V	VBL + DAS	7	14.39 ± 0.7	73[a]
VI	VBL + VPL	8	15.26 ± 0.9	71[a]
VII	VCR	24	47.95 ± 4.3	
VIII	VCR + DAS	9	17.88 ± 0.8	65[b]
IX	VCR + VPL	9	17.33 ± 1.3	64[b]

[a]Inhibition (%) was calculated over group IV.
[b]Inhibition (%) was calculated over group VII.

patient. 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.

Acknowledgements

The authors are thankful to Prof. Y.K.Gupta (Director, Industrial Toxicology Research Centre, Lucknow) for his keen interest and support during the course of this study. Thanks are also due to Dr Neeraj Mathur (Scientist) for statistically analyzing the data. The authors are also thankful to the Council of Scientific & Industrial Research (New Delhi) for providing a Senior Research Fellowship to A.A. The authors also acknowledge DST (New Delhi) for providing a Women Scientist (WOS) to K.S.

References

1. Gottesman,M.M. and Pastan,I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.*, **62**, 385–427.
2. Malayeri,R., Filipits,M., Suchomel,R.W., Zochbauer,S., Lechner,K. and Pirker,R. (1996) Multidrug resistance in leukemias and its reversal. *Leuk. Lymph.*, **23**, 451–458.
3. Ross,D.D., Wooten,P.J., Sridhara,R., Ordonez,J.V., Lee,E.J. and Schiffer,C.A. (1993) Enhancement of daunorubicin accumulation, retention and cytotoxicity by verapamil or cyclosporin A in blast cells from patients with previously untreated acute myeloid leukaemia. *Blood*, **82**, 1288–1299.
4. Fan,D., Beltran,P.J. and O'Brian,C.A. (1994) In Kellen,J.A. (ed.), *Reversal of Multidrug Resistance in Cancer*. CRC Press, Boca Raton, FL, pp. 93–125.
5. Ambudkar,A.S.V., Dey,S., Hrycyna,C.A., Ramachandra,M., Pastan,I. and Gottesman,M.M. (1999) Biochemical, cellular and pharmacological aspects of the multidrug transporter. *Annu. Rev. Pharmacol. Toxicol.*, **39**, 361–398.
6. Sugawara,I. (1990) Expression and functions of P-glycoprotein (MDR-1 gene product) in normal and malignant tissues. *Acta Pathol. Jpn*, **40**, 545–553.
7. Sikic,B.I., Fisher,G.A., Lum,B.L., Halsey,J., Beketic-Oreskovic,L. and Chen,G. (1997) Modulation and prevention of multidrug resistance by inhibitors of P-glycoprotein. *Cancer Chemother. Pharmacol.*, **40**, 13–19.
8. Watanabe,T., Tsuge,H., Oh-Hara,T., Saito,M. and Tsuruo,T. (1995) Comparative study on reversal efficacy of SDZ PSC 833, cyclosporin A and verapamil on multidrug resistance *in-vitro* and *in-vivo*. *Acta Oncol.*, **34**, 235–241.

9. Mechetner,E.B. and Roninson,I.B. (1992) Efficient inhibition of P-glycoprotein mediated multidrug resistance with a monoclonal antibody. *Proc. Natl Acad. Sci. USA*, **89**, 5824–5828.
10. Thierry,A.R., Rahman,A. and Dritschilo,A. (1993) Overcoming multidrug resistance in human tumour cells using free and liposomaly encapsulated antisense oligodeoxy-nucleotides. *Biochem. Biophys. Res. Commun*., **190**, 952–960.
11. Ford,J.M. and Hait,W.N. (1990) Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol. Rev*., **42**, 155–199.
12. Pirker,R., Zochbauer,S., Kupper,H., Lassmann,A., Gsur,A., Frass,M., Krajnik,G., Knobl,P. and Lechner,A. (1995) Dexvirapamil as resistance modifier in acute myeloid leukaemia. *J. Cancer Res. Clin. Oncol*., **121** (suppl. 3), R21–R24.
13. Slate,L.D., Bruno,A.N., Casey,M.S., Zutshi,N., Garvin,J.L., Wu,H. and Pfister,R.J. (1995) RS-33295-198: a novel, potent modulator of P-glycoprotein-mediated multidrug resistance. *Anticancer Res*., **15**, 811–814.
14. Pinto,J.J. and Rivlin,R.S. (2001) Antiproliferative effects of allium derivatives from garlic. *J. Nutr*., **131**, 1058s–1060s.
15. Le Bon,A.M., Roy,C., Dupont,C. and Suschetet,M. (1997) *In vivo* antigenotoxic effects of dietary allyl sulfides in the rat. *Cancer Lett*., **114**, 131–14.
16. Agarwal,K.C. (1996) Therapeutic actions of garlic constituents. *Med. Res. Rev*., **16**, 111–124.
17. Fukushima,S., Takada,N., Hori,T. and Wanibuchi,H. (1997) Cancer prevention by organosulfur compounds from garlic and onion. *J. Cell. Biochem*., **27** (suppl.), 100–105.
18. Yang,C.S., Chhabra,S.K., Hong,J.Y. and Smith,T.J. (2001) Mechanisms of inhibition of chemical toxicity and carcinogenesis by diallyl sulfide (DAS) and related compounds from garlic. *J. Nutr*., **131**, 10415–10455.
19. Riggs,D.R., DeHaven,J.I. and Lamm,D.L. (1997) *Allium sativum* (garlic) treatment for murine transitional cell carcinoma. *Cancer*, **79**, 1987–1994.
20. Hong,Y.S., Ham,Y.A., Choi,J.H. and Kim,J. (2000) Effects of allyl sulfur compounds and garlic extract on the expression of Bcl-2, Bax and p-53 in non-small cell lung cancer cell lines. *Exp. Mol. Med*., **32**, 127–134.
21. Wargovich,M.J., Imada,O. and Stephens,L.C. (1992) Initiation and post initiation chemopreventive effects of diallyl sulfide in esophageal carcinogenesis. *Cancer Lett*., **64**, 39–42.
22. Singh,A. and Shukla,Y. (1998) Antitumour activity of diallyl sulfide on polycyclic aromatic hydrocarbon induced mouse skin carcinogenesis. *Cancer Lett*., **131**, 209–214.
23. Singh,A. and Shukla,Y. (1998) Antitumour activity of diallyl sulfide in two stage mouse skin model of carcinogenesis. *Biomed. Environ. Sci*., **11**, 258–263.
24. Shukla,Y., Arora,A. and Singh,A. (2002) Antitumourigenic potential of diallyl sulfide in Ehrlich ascites tumour bearing mice. *Biomed. Environ. Sci*., **15**, 41–47.
25. Arora,A. and Shukla,Y. (2003) Induction of apoptosis by diallyl sulfide in DMBA-induced mouse skin tumors. *Nutr. Cancer*, **44**, 89–93.
26. Haber-Mignard,D., Suschetet,M., Berges,A., Astorg,P. and Siess,M.H. (1996) Inhibition of aflatoxin B_1 and N-nitrosodiethylamine induced liver preneoplastic foci in rats fed naturally occurring allyl sulfides. *Nutr. Cancer*, **25**, 61–70.
27. Lozzio,C.B. and Lozzio,R.B. (1975) Human chronic myelogenous leukaemia cell line with positive Philadelphia chromosome. *Blood*, **45**, 321–324.
28. Mosmann,T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J. Immunol. Methods*, **65**, 55–63.
29. Towbin,H., Staehelin,T. and Gordon,J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA*, **76**, 4350–4354.
30. Lowry,O.H., Rosenbrough,N.K., Farr,A.L. and Randall,R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem*., **193**, 265–275.
31. Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–682.
32. Gottesman,M.M. (1993) How cancer cells evade chemotherapy. *Cancer Res*., **53**, 747–754.
33. Pastan,I. and Gottesman,M.M. (1993) Multiple drug resistance in human cancers. *N. Engl. J. Med*., **316**, 385–427.
34. Roninson,I.B. (1992) The role of MDR-1 (P-glycoprotein) gene in multidrug resistance *in-vitro* and *in-vivo*. *Biochem. Pharmacol*., **43**, 95–102.
35. Tsuruo,T., Oh-Hara,T. and Saito,H. (1986) Characteristics of vincristine resistance in vincristine resistant human myelogenous leukaemia K562. *Anticancer Res*., **6**, 637–641.
36. Tan,B., Piwnica-Worms,D. and Ratner,L. (2000) Multidrug resistance transporters and modulation. *Curr. Opin. Oncol*., **12**, 450–458.
37. Robert,J. (1997) Multidrug resistance reversal agents. *Drugs Future*, **22**, 149–158.
38. Arora,A. and Shukla,Y. (2003) Modulation of P-glycoprotein mediated multidrug resistance by indole-3-carbinol. *Cancer Lett*., **189**, 167–173
39. Christensen,J.C. and LeBlanc,G. (1996) Reversal of multidrug resistance *in vivo* by dietary administration of the phytochemical indole-3-carbinol. *Cancer Res*., **56**, 574–581
40. Zhu,A., Wang,X. and Guo,Z. (2001) Study of tea polyphenols as a reversal agent for carcinoma cell lines' multidrug resistance (study of TP as a MDR reversal agent). *Nucl. Med. Biol*., **28**, 735–748.
41. Sadzuka,Y., Sugiyama,T. and Sonobe,T. (2000) Efficacies of tea components on doxorubicin induced antitumor activity and reversal of multidrug resistance. *Toxicol. Lett*., **114**, 155–162.
42. Ishikawa,M., Fujita,R., Takayanagi,Y. and Sasaki,K. (2000) Reversal of acquired resistance to doxorubicin in K562 human leukemia cells by astemizole. *Biol. Pharm. Bull*., **23**, 112–115.
43. Zamora,J.M., Pearce,H.L. and Beck,W.T. (1988) Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemia cells. *Mol. Pharmacol*., **33**, 454–462.
44. Brady,J.F., Ishizaki,H., Fukuto,J.M., Lin,M.C., Fadel,A., Gapac,J.M. and Yang,C.S. (1991) Inhibition of cytochrome P-450 2E1 by diallyl sulfide and its metabolites. *Chem. Res. Toxicol*., **4**, 642–647.
45. Jin,L. and Baillie,T.A. (1997) Metabolism of the chemoprotective agent diallyl sulfide to glutathione conjugates in rats. *Chem. Res. Toxicol*., **10**, 318–327.

Received August 6, 2003; revised December 11, 2003; accepted December 12, 2003