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Prevention of adriamycin-induced *mdr1* gene amplification and expression in mouse leukemia cells by simultaneous treatment with the anti-recombinogen bromovinyldeoxyuridine

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Summary

The anti-recombinogenic substance (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) was tested for its ability to prevent adriamycin-induced *mdr1* gene amplification and expression in mouse leukemia cells *in vitro*. F4-6 cells that were treated with stepwise enhanced doses of adriamycin acquired resistance against adriamycin. While 20 ng/ml adriamycin showed strong toxic effects in sensitive cells, the same dose was tolerated at the end of the long-term experiment following treatment with stepwise enhanced doses of adriamycin. In parallel experiments, 0.5 or 1 μg/ml BVDU was given together with adriamycin. BVDU prevented the formation of resistance against adriamycin treatment. Using differential PCR, the signal intensity of the *mdr1a*-specific band appeared markedly increased in adriamycin-resistant cells, while the signal intensities of the adriamycin + BVDU-treated cells resembled the intensity ratio of the untreated control cells. Beyond that, in resistant F4-6 cells increased expression of *mdr* genes was demonstrated by Northern blot analysis.

Key words

gene amplification/leukemia/mouse cells/multidrug resistance/recombination

Development of multidrug resistance (MDR) in hamster, mouse and human cell lines is frequently associated with *mdr* gene amplification, and the level of drug resistance is often correlated with the level of gene amplification (Gottesman, 1993). *mdr* gene amplification in association with multidrug resistance in mice and rats has been observed not only *in vitro*, but also *in vivo*. Mouse leukemia cells, selected *in vivo* for resistance to anthracyclines, have developed *mdr* gene amplification (Schimke *et al.*, 1986; Demidova *et al.*, 1987; Gudkov *et al.*, 1991; Volm *et al.*, 1991).

As it is generally agreed that the first steps of gene amplification and recombination are the same (Schimke *et al.*, 1986; Volkert and Broach, 1986; Wahl, 1989), both processes may be causally related. To test this hypothesis, in previous experiments co-recombinogens and anti-recombinogens were studied for their ability to enhance or suppress the cytostatica-induced formation of SV40 amplification. There could be observed a very strong correlation between both effects (Fahrig and Steinkamp-Zucht, 1996). (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU) was the only substance within the group of anti-recombinogens with likely clinical significance (Fahrig, 1996). Therefore, this substance was selected for the present *in vitro* experiments using a mouse tumor cell line that is known to develop adriamycin resistance corresponding to *mdr* gene amplification. F4-6 tumor cells are Friend virus-transformed mouse erythroleukemia cells (Ostertag *et al.*, 1972). Cell line F4-6wt shows the wild type, i.e. is drug sensitive and has no increased *mdr* gene expression and amplification.

F4-6 cells were treated with stepwise enhanced doses of adriamycin. In the course of these experiments the cell population acquired resistance against adriamycin. While 20 ng/ml adriamycin showed strong toxic effects in sensitive cells, the same dose was tolerated at the end of the long-term experiment. In parallel experiments, adriamycin was given together with 0.5 or 1 μg/ml BVDU (Figure 1A). At these doses, BVDU had no toxic effects when given alone (Figure 1B). It is apparent that BVDU prevented the formation of resistance against adriamycin treatment. The tumor cells remained sensitive against adriamycin treatment.

In particular, 10^5 F4-6 cells were seeded into 5 ml DMEM (Dulbecco's minimum essential medium). Four days (86 h) later, the medium was replaced by fresh medium, and

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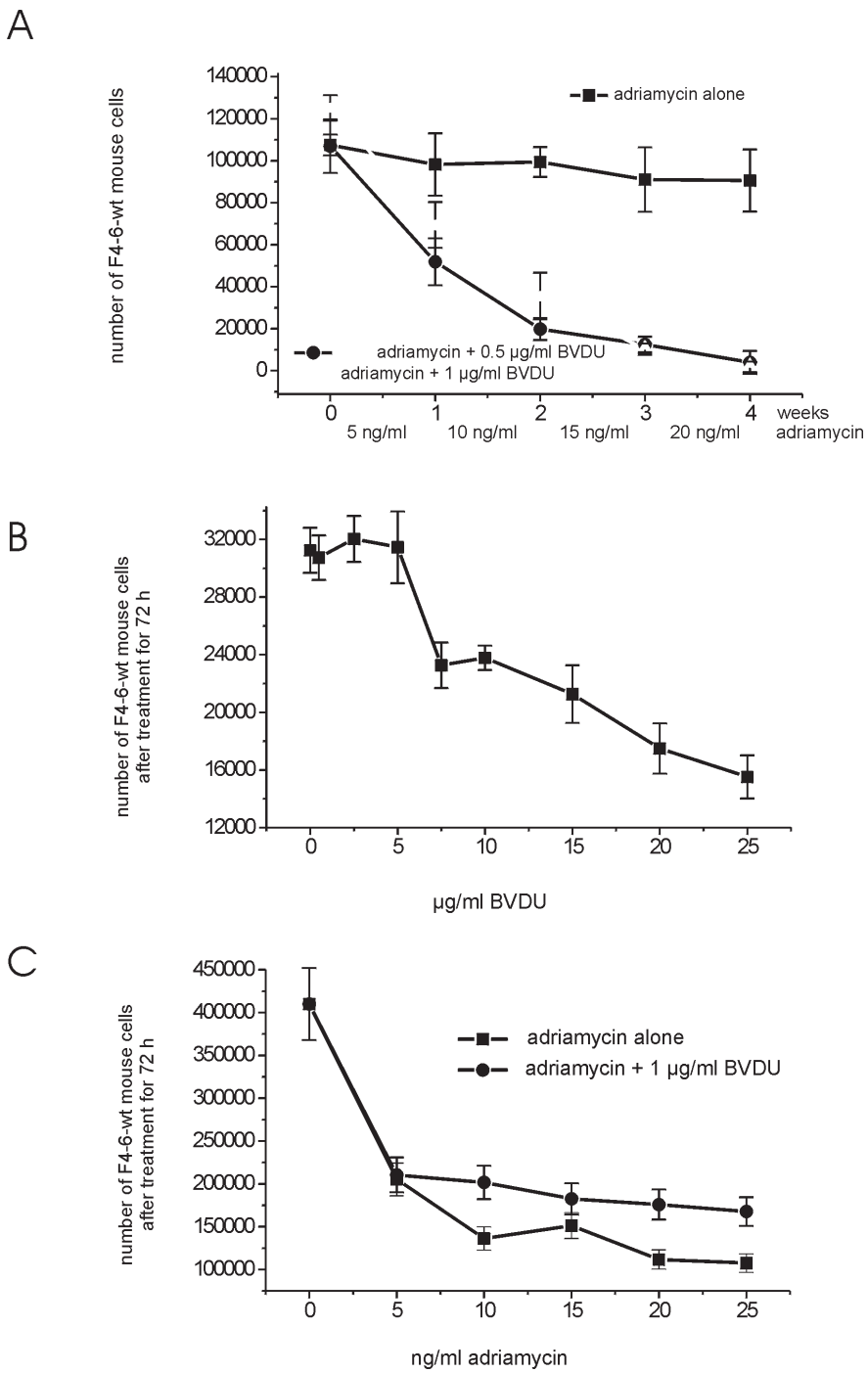

Figure 1

(A) Treatment of mouse erythroleukemia F4-6-wt cells (drug sensitive, no expression of *mdr* genes) for 4 weeks with stepwise enhanced doses of adriamycin, or adriamycin + BVDU. (B) Treatment of mouse erythroleukemia F4-6-wt cells (drug sensitive, no expression of *mdr* genes) for 72 h with different doses of BVDU. (C) Treatment of mouse erythroleukemia F4-6-wt cells (drug sensitive, no expression of *mdr* genes) for 72 h with different doses of adriamycin, or adriamycin + 1 µg/ml BVDU.

adriamycin alone, BVDU alone or BVDU + adriamycin were added to the cultures. After 72 h of treatment the medium was replaced by fresh medium and the same or higher concentrations of the substances were added. The toxic effect of adriamycin was so strong that the treatment had to be interrupted from time to time for 4 days where the cells were allowed to grow without treatment. In contrast to this, the growth curves for BVDU (0.5 and 1 µg/ml) were similar to those of the untreated control. Therefore, the cells of these cultures had to be serially passaged.

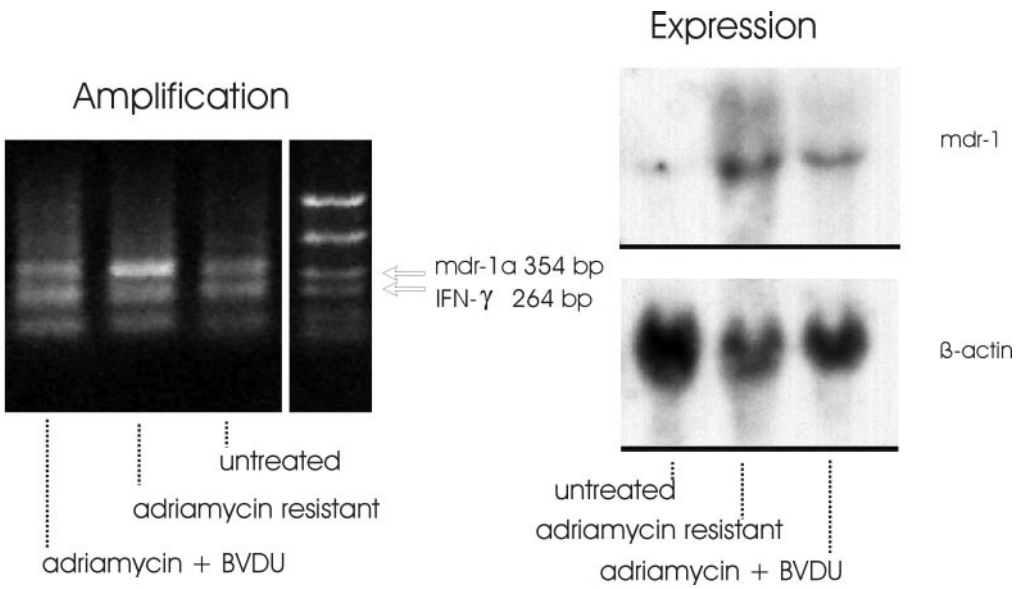

Figure 2

(Right) Detection of *mdr1* gene expression by Northern blot analysis of RNA: F4-6-wt (wild-type) cells. F4-6 cells, resistant to adriamycin after treatment with stepwise enhanced doses of adriamycin. F4-6-wt cells, not resistant to adriamycin after treatment with stepwise enhanced doses of adriamycin in combination with BVDU. (Left) Detection of amplified *mdr1a* gene by differential PCR: F4-6-wt (wild-type) cells; F4-6 adriamycin-resistant cells; and F4-6-wt cells treated for 4 weeks with stepwise enhanced doses of adriamycin up to a dose of 20 ng/ml in combination with 1 μg/ml BVDU.

To determine the number of living cells, a CASY 1 cell counter and analyzer system (Winkelmeier *et al*., 1993) was used. Cell counting and volume determination is based on the displacement of conductive electrolyte by dielectric cells. The signals generated by the cells suspended in an electrolyte are evaluated by pulse area analysis. The pulse area of the signal is strictly proportional to the volume of the particle generating the signal. In dead cells the integrity of the cell membrane is lost. This loss increases the conductivity and reduces the pulse area of the electric signal. Thus, to exclude debris and dead cells, only particles with a size of 6–10 μm were counted as vital cells.

To ensure that *mdr* gene amplification is involved during the treatment of F4-6 cells, we performed a differential polymerase chain reaction (PCR) as described by Frye *et al.* (1989). The method is based on a simultaneous PCR amplification of a single copy gene together with the gene of interest (here *mdr1a*). It has been shown that the PCR product yield (i.e. differences in signal intensities) is preserved during later cycles with differential PCR but not with conventional PCR. Interferon-γ was used as a single copy reference gene. The DNA from ~1 × 10^6 cells was purified with the 'InViSorb' genomic DNA purification kit (InViTek, Berlin, Germany). The PCR mixtures (reaction volume 50 μl) contained the target DNA sample, 200 pM of each primer, 1 Unit *Taq* polymerase, 0.25 mM of each dNTP and a reaction buffer containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3). Aliquots of the genomic DNA were amplified as follows: initial denaturation at 99°C for 3 min, initial annealing with addition of *Taq* polymerase at 52°C for 4 min. The following 35 cycles included 30 s of denaturation and annealing (96°C, 52°C) and an extension at 72°C for 2.5 min except for the final extension step, which was for 12.5 min. PCR reactions were performed using four different primers: MMIFN1:5′-GTGCTGCCGTCATTTTCTGC-3′, MMIFN2: 5′-CCCTTTCTTCCCTTC TTCGTTC-3′, MMMDR1A-3: 5′-GGTGGATATGGGGGACTTTTGG TA-3′, MMDR1A-4: 5′-CCCCGTGCAGGCTTTTCTTCA-3′.

As a result of these experiments, it can be stated that the signal intensity of the *mdr1a*-specific band appeared markedly increased in adriamycin-resistant cells. The signal intensities of the adriamycin + BVDU-treated cells resembled the intensity ratio of the untreated control cells (Figure 2, left).

The expression of the *mdr1* gene was studied in adriamycin-sensitive and -resistant F4-6 cells selected using Northern blot analysis. Levels of β-actin mRNA were also analyzed comparatively. β-Actin was used as an internal control for equivalent amounts of RNA loaded. Total cellular RNA from drug-sensitive and -resistant F4-6 cells was isolated by the method of Chomczynski and Sacchi (1987). RNA (30 μg) was denatured by glyoxalation, electrophoresed through a 1% agarose gel and transferred to Biodine A membrane (PALL, Portsmouth, UK) by the capillary blotting technique using 20 × SSD solution (1 × SSD contains 0.15 M sodium chloride and 0.015 M sodium citrate). RNA was immobilized by baking the membrane at 80°C for 1 h. DNA probes for hybridization were labeled

Table I

Comparison of IC_{50} and the corresponding concentrations ($n = 3 \pm$ SE)

	Untreated control	*Adriamycin (ng/ml)*	*BVDU (μg/ml)*	*Adriamycin + BVDU*
F4-6-wt (wild-type) cells				
IC_{50}	–	5	25	10 + 1
No. of viable cells	410 000 ± 39 250	205 250 ± 19 125	–	201 750 ± 19 375
	31 250 ± 1562	–	15 525 ± 1500	–
F4-6 cells, resistant to 20 ng/ml adriamycin after treatment with stepwise enhanced doses of adriamycin				
IC_{50}	–	35	–	35 + 1
No. of viable cells	305 750 ± 29 250	140 500 ± 13 900	–	160 250 ± 14 500

with [α-^{32}P]dCTP by the random primer method using the Multiprime DNA labeling system from Amersham (Braunschweig, Germany). Membrane hybridization was carried out after 6 h prehybridization as described by Braun *et al.* (1989). Hybridized blots were washed in 2 × SSD and 0.1% SDS at room temperature for 20 min and at 50°C for 60 min, in 0.5 × SSC and 0.1% SDS at 50°C, and finally in 0.2 × SSC and 0.1% SDS at 55°C for 30 min. The membranes were exposed at –80°C to an X-ray film (Fuji Photo Film, Tokyo, Japan) using intensifying screens. The membranes were sequentially hybridized with the DNA probes. The following probes were used for hybridization: human *mdr1* probe pHDR5A, 1.38 kb, and human β-actin cDNA probe, 1.2 kb.

In resistant F4-6 cells increased expression of *mdr* genes (Figure 2, right), and in wild-type adriamycin-sensitive F4-6 cells *mdr* transcripts at scarcely detectable levels could be clearly demonstrated. This position corresponds to the size of the *mdr1b* mRNA (Hsu *et al.*, 1989). In comparison, in the adriamycin-resistant cells, transcripts ranging from 4.5 to 6 kb reacted very strongly with the *mdr1* cDNA probe. As discussed previously (Schaefer *et al.*, 1993), the combined message obviously results from the crossreaction of the human *mdr1* cDNA probe with the mouse *mdr1a* and *mdr1b* mRNAs and from the heterogeneity in the size of *mdr1a* transcripts (Hsu *et al.*, 1989, 1990).

It is apparent that BVDU + adriamycin treatment led to *mdr* transcripts at more than five times lower levels than treatment with adriamycin alone, and that BVDU inhibited the formation of *mdr gene* amplification. In reality the preventative effect of BVDU was much higher than is expressed in the lower levels of *mdr* transcripts because the cells that did not undergo *mdr* gene amplification were killed during treatment and could not be detected. At the end of treatment only cells survived that were resistant at least to a certain extent. Therefore, the more relevant effect is the inactivation of cells seen in Figure 1A.

The effectiveness of DNA replication or repair of DNA damage depends on a balanced supply of deoxyribonucleoside triphosphate (dNTP) precursors of DNA. Should cellular dNTP levels be perturbed, a wide range of genetic events may follow due to aberrant DNA replication or repair. For example, manifestations of DNA precursor imbalance can include gene mutation; recombination; enhanced sensitivity to mutagens/carcinogens (i.e. co-mutagenic and co-carcinogenic effects); and chromosome breakage, exchange or loss (Haynes, 1985; Kunz *et al.*, 1994). Therefore, thinking about the mode of action of 5-substituted pyrimidine nucleoside analogs, possible changes in nucleotide pools have to be taken into account. It seems evident that for the repair of cytostatica-induced DNA damage this pool could be very important, and that the addition of 5-substituted pyrimidine nucleoside analogs could cause modifications not only in the available pools but also by changing the fidelity of certain polymerases. Such changes could influence recombination as well as gene amplification.

Theoretically it is possible that BVDU, by disrupting nucleotide pools or inhibiting key enzymes, might act as general inhibitor of DNA repair. As such, inhibition of recombination may be only one outcome. BVDU may also act to increase the cytotoxicity of chemotherapeutic agents by blocking repair of induced DNA lesions.

To demonstrate that BVDU does not simply act as a general inhibitor of DNA repair just increasing the cytotoxicity of adriamycin by blocking repair of adriamycin-induced DNA lesions, F4-6-wt-sensitive cells were treated for 72 h with up to 25 ng/ml adriamycin alone or with adriamycin + 1 μg/ml BVDU. It can be seen in Figure 1C and Table I in respect to the IC_{50} that BVDU did not enhance the effect of adriamycin. On the contrary, the toxic effect of adriamycin seems to be lowered. This reduction of toxicity could also be observed *in vivo*: tumor-bearing rats treated with adriamycin alone showed a strong loss of body weight at longer treatment times. Rats treated with adriamycin (3 × 4 mg/kg per week) + BVDU (5×15 mg/kg per week) showed an increase in body weight and decrease in tumor weight (unpublished results).

Drugs that reverse MDR have been shown to inhibit the P-glycoprotein-mediated drug efflux mechanism (Ford and Hait, 1990). Therefore, another question was whether it is possible to circumvent multidrug resistance by treatment of resistant tumor cells (overexpressing the *mdr* gene) with BVDU. F4-6 cells (resistant to 20 ng/ml adriamycin), col-

lected at the end of the 4 week experiment, were treated for 72 h with 35 ng/ml adriamycin alone, or with adriamycin + 1 μg/ml BVDU. It is apparent (Table I) that BVDU did not influence the effect of adriamycin. The IC_{50} was similar for both treatments.

Although not directly proven, virtually all proposed mechanisms for gene amplification involve recombination (Schimke *et al.*, 1986; Volkert and Broach, 1986; Wahl, 1989). In fact, it is difficult to think of any model for gene amplification that would not involve recombination. Therefore, it was of interest to see if the anti-recombinogenic effect of BVDU in yeast (Fahrig, 1996), which had been confirmed by the anti-amplification effect on SV40 DNA in Chinese hamster cells (Fahrig and Steinkamp-Zucht, 1996), could be observed also in a relevant mammalian gene. As this was the result of our study, the idea is supported that gene amplification is mechanistically related to recombination. In contrast to those drugs used for reducing the function of P-glycoprotein, the action of BVDU is directed against the mechanism of resistance, i.e. amplification of several target genes may be attacked, causing many neoplastic cells to develop resistance to anticancer drugs. We have shown that *mdr* gene amplification and expression could be detected in adriamycin-treated and -resistant cells *in vitro*, underlining the hypothesis that gene amplification is part of the mechanism whereby these cells gain resistance against antineoplastic drugs.

Chronic treatment with antineoplasic drugs leads not only to amplification of the *mdr* gene, but also to the overexpression and amplification of certain oncogenes controlling resistance to this treatment. The results of Bhushan *et al.* (1992) are consistent with the hypothesis that induction of *c-fos* by low levels of cytotoxic drugs may be an early event in the acquisition of the MDR phenotype. Furthermore, Bhuschan *et al.* (1996) could show that enhanced expression of the *c-fos* gene preceded the expression of the *mdr3* gene. In MDR sublines of both murine sarcoma and human KB-3-1 carcinoma cell lines, the expression of *c-fos* and *c-jun* increased as drug resistance increased. Nakagawara *et al.* (1990) found an inverse correlation between expression of amplified N-*myc* and the expression of *mdr1* in the same human neuroblastoma tumor samples. Neuroblastomas with N-*myc* amplification no longer responded to chemotherapy. Bordow *et al.* (1994) and Norris *et al.* (1996) observed that expression of the multidrug resistance-associated membrane-bound glycoprotein (MRP) gene correlated with amplification and overexpression of the N-*myc* oncogene in childhood neuroblastoma, which is central to the malignant phenotype of this disease. Therefore, gene amplification may be a central mechanism in acquiring chemoresistance.

Our *in vitro* study with adriamycin + BVDU has been reconfirmed *in vivo* in our laboratory and in an industrial laboratory. BVDU inhibited at plasma levels that were similar to those in the *in vitro* experiments of adriamycin-induced amplification and expression of the *mdr1* gene in tumors of rats *in vivo*. Through this inhibition of gene amplification, BVDU strongly enhanced the effects of adriamycin in suppressing the growth of fibrosarcomas, mammary adenocarcinomas and Ah13 sarcomas. We are currently examining the effects of BVDU *in vivo* in combination with different cytostatic drugs. Resistance against these drugs involves the amplification/expression of genes other than the *mdr1* gene.

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References

Bhushan A., Abramson R., Chiu J.F., Tritton T.R. (1992) Expression of c-fos in human and murine multidrug-resistant cells. *Molecular Pharmacology*, **42**, 69.

Bhushan A., Slapak C.A., Levy S.B., Tritton T.R. (1996) Expression of c-fos precedes MDR3 in vincristine and adriamycin selected multidrug resistant murine erythroleukemia cells. *Biochemistry and Biophysics Research Communications*, **226**, 819.

Bordow S.B., Haber M., Madafiglio J., Cheung B., Marshall G.M., Norris M.D. (1994) Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the *N-myc* oncogene in childhood neuroblastoma. *Cancer Research*, **54**, 5036.

Braun T., Bober E., Buschhausen-Denker G., Kotz S., Grzeschik K.-H., Arnold H.H. (1989) Differential expression of miogenic determination genes in muscle cells: possible autoactivation by the myf gene products. *EMBO Journal*, **8**, 3617.

Chomczynski P., Sacchi N. (1987) Single-step method of DNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry*, **162**, 156.

Demidova N.S., Goncharova S.A., Chernova O.B., Kopnin B.P., Gudkov A.V. (1987) Gene amplification in murine leukemia cells with multiple drug resistance acquired *in vivo*. *Genetika*, **23**, 1797.

Fahrig R. (1996) Anti-recombinogenic and convertible

co-mutagenic effects of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and other 5-substituted pyrimidine nucleoside analogs in *S. cerevisiae* MP1. *Mutation Research*, **372**, 133.

Fahrig R., Steinkamp-Zucht A. (1996) Induction or suppression of SV40 amplification by genotoxic carcinogens, non-genotoxic carcinogens or tumor promoters. *Mutation Research*, **356**, 217.

Ford J.M., Hait W.N. (1990) Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacology Review*, **42**, 155.

Frye R.A., Benz C.C., Edison L. (1989) Detection of amplified oncogenes by differential polymerase chain reaction. *Oncogene*, **4**, 1153.

Gottesman M.M. (1993) How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal foundation award. *Cancer Research*, **53**, 747.

Gudkov A.V., Chernova O.B., Kopnin B.P. (1991) Karyotype and amplicon evolution during stepwise development of multidrug resistance in Djungarian hamster cell lines. In *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells*. Roninson I.B. (ed.), p. 147. Plenum: New York.

Haynes R.H. (1985) Molecular mechanisms in genetic stability and change: the role of deoxyribonucleotide pool balance. In *Genetic Consequences of Nucleotide Pool Imbalance*. de Serres F.J. (ed.), p. 1. Plenum: New York.

Hsu S.I.-H., Lothstein L., Horwitz S.B. (1989) Differential overexpression of the mdr genes family members in multidrug-resistant J 774.2 mouse cells. Evidence that distinct P-glycoprotein precursors are encoded by unique mdr genes. *Journal of Biological Chemistry*, **264**, 12053.

Hsu S.I.-H., Cohen D., Kirschner L.S., Lothstein L., Hartstein M., Horwitz S.B. (1990) Structural analysis of the mouse mdr1a (P-glycoprotein) promoter reveals the basis of differential transcript heterogeneity in multidrug-resistant J 774.2 cells. *Molecular Cell Biology*, **10**, 3596.

Kunz B.A., Kohalmi S.E., Kunkel T.A., Mathews C.K., McIntosh E.M., Reidy J.A. (1994) Deoxyribonucleoside triphosphate levels: a critical factor in the maintenance of genetic stability. *Mutation Research*, **318**, 1.

Nakagawara A., Kadomatsu K., Shin-Ichi S., Kohno K., Akazawa K., Nose Y., Kuwano M. (1990) Inverse correlation between expression of multidrug resistance gene and N-*myc* oncogene in human neuroblastomas. *Cancer Research*, **50**, 3043.

Norris M.D., Bordow S.B., Marshall G.M., Haber P.S., Cohn S.L., Haber M. (1996) Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma. *New England Journal of Medicine*, **334**, 231.

Ostertag W., von Ehrenstein G., Charache S. (1972) Duplicated-chain genes in Hopkins-2 haemoglobin of man and evidence for unequal crossing over between them. *Nature New Biology*, **237**, 90.

Schaefer A., Westendorf J., Lingelbach K., Schmidt C.A., Mihalache D.-L., Reymann A., Marquardt H. (1993) Decreased resistance to *N*,*N*-dimethylated anthracyclines in multidrug-resistant Friend erythroleukemia cells. *Cancer Chemotherapy and Pharmacology*, **31**, 301.

Schimke R.T., Sherwood S.W., Hill A.B., Johnston R.N. (1986) Overreplication and recombination of DNA in higher eukaryotes: potential consequences and biological implications. *Proceedings of the National Academy of Sciences, USA*, **83**, 2157.

Volkert F.C., Broach J.R. (1986) Site-specific recombination promotes plasmid amplification in yeast. *Cell*, **46**, 541.

Volm M., Mattern J., Pommerenke E.W. (1991) Time course of mdr gene amplification during *in vivo* selection for adriamycin-resistance and during reversal in murine leukemia L 1210. *Anticancer Research*, **11**, 579.

Wahl G.M. (1989) The importance of circular DNA in mammalian gene amplification. *Cancer Research*, **49**, 1333.

Winkelmeier P., Glauner B., Lindl T. (1993) Quantification of cytotoxicity by cell volume and cell proliferation. *Alternative to Laboratory Animals: ATLA*, **21**, 269.