Jpn. J. Cancer Res. 82, 351–356, March 1991

Enhancement by Verapamil of Neocarzinostatin Action on Multidrug-resistant Chinese Hamster Ovary Cells: Possible Release of Nonprotein Chromophore in Cells

Yoichi Miyamoto and Hiroshi Maeda[1]

Department of Microbiology, Kumamoto University Medical School, 2-2-1 Honjo, Kumamoto 860

Multidrug-resistant CH^RC5 cells were about 10-fold more resistant to the proteinaceous anticancer drug neocarzinostatin (NCS) and its nonprotein chromophore (NPC) than the parental AUXB1 cells. There was little difference in cell growth, glutathione content, or activities of several antioxidant enzymes between the two cell lines. The degree of intracellular incorporation and extracellular excretion of fluorescein isothiocyanate-labeled NCS by CH^RC5 cells was similar to that of AUXB1 cells. On the other hand, 20 μM verapamil or 27 μM cepharanthine restored the susceptibility of CH^RC5 cells to NCS and NPC to the level of AUXB1 cells. In addition, NPC was found to suppress the photolabeling of [^{3}H]azidopine (a known P-glycoprotein-binding ligand) to plasma membranes of CH^RC5 cells. All these findings favor the possibility that NPC was excreted via P-glycoprotein, which may contribute to the resistance of CH^RC5 cells to NCS.

Key words: Multidrug resistance — Neocarzinostatin — Nonprotein chromophore — Verapamil

A macromolecular anticancer drug, neocarzinostatin (NCS),[2] exhibited cytotoxicity against two different types of Chinese hamster ovary cells, parental AUXB1 and its multidrug-resistant subline, CH^RC5 cells, at nanomolar concentrations,[1] as was shown for other cell lines.[2] CH^RC5 cells were 20- to 900-fold more resistant than AUXB1 cells to adriamycin, aclacinomycin, vinblastine, and mitomycin C. CH^RC5 cells were also about 10-fold more resistant than AUXB1 cells to NCS.[1] The increased active efflux of anthracyclines and vinca alkaloids was correlated with the multidrug resistance in many cell lines,[3,4] including CH^RC5 cells.[5]

We previously reported that macromolecular agents were taken up into cells by an endocytotic mechanism[6-9] and more recently that the efflux mechanisms for low-molecular-weight and high-molecular-weight agents were different: the former agents are more vulnerable to the P-glycoprotein-dependent efflux mechanism. Few reports are available on the development of resistance to NCS, and we thus attempted to elucidate the cellular mechanism of resistance to NCS in CH^RC5 cells. The cytotoxic action of NCS resides in a labile nonprotein chromophore (NPC), which undergoes a rapid and irreversible reaction with thiols to produce a radical species that interacts with and cleaves DNA.[10] However, there is no report to our knowledge as to the intracellular liberation of NPC.

Therefore, we examined the cell growth rate, antioxidant defense systems, drug influx/efflux, and the effects of P-glycoprotein inhibitors on sensitivity to NCS and NPC, and further examined the effect of NPC on the photolabeling with [^{3}H]azidopine of plasma membranes of CH^RC5 cells, to clarify the mechanism of resistance of the cells to NCS.

MATERIALS AND METHODS

Cell lines An auxotrophic mutant of Chinese hamster ovary cells, AUXB1, and its multidrug-resistant subline expressing P-glycoprotein at a high level, CH^RC5, were generous gifts from Professor Victor Ling, Ontario Cancer Institute, Toronto, Ontario, Canada.[5] These cell lines were cultured in Stanner's modified minimal essential medium supplemented with antibiotics and 10% fetal bovine serum.

Drugs and chemicals NCS (M_r 12,000) was obtained from Kayaku Antibiotics Research Laboratories, Tokyo. NPC was extracted from NCS powder with acetic acid according to the method previously described[11] and was stored at −70°C. [^{3}H]Azidopine (52 Ci/mmol) was obtained from Amersham Japan, Tokyo. Fluorescein isothiocyanate-labeled NCS (F-NCS) was prepared by essentially the same method as previously described.[12] All other chemicals were from commercial sources.

---

[1] To whom correspondence should be sent.

[2] Abbreviations used: NCS, neocarzinostatin; NPC, nonprotein chromophore; BSA, bovine serum albumin; SMANCS, styrene-maleic acid copolymer-conjugated NCS; FITC, fluorescein isothiocyanate; F-NCS, FITC-labeled NCS; F-BSA, FITC-labeled BSA; F-SMANCS, FITC-labeled SMANCS; KRP, Krebs-Ringer phosphate buffer; IC_{50}, 50% inhibitory concentration; GSH, glutathione; NPSH, nonprotein sulfhydryls; GPX, glutathione peroxidase; GST, glutathione-*S*-transferase; Cu,Zn-SOD, Cu,Zn-containing superoxide dismutase; Mn-SOD, Mn-containing superoxide dismutase; SDS, sodium dodecyl sulfate.

Cytotoxicity study Cytotoxicity was assayed in terms of inhibition of colony formation.[1] The cells were plated in a 96-well flat-bottomed plate (Falcon, no. 3072) at a density of 3×10^4 cells/well and were cultured overnight. After removal of the medium, cells were exposed to various concentrations of drugs in growth medium for 24 h at 37°C. Then the drug-containing medium was removed, the cells in each well were trypsinized and diluted with the growth medium, and about 500 cells were seeded into dishes (Falcon, no. 3001). After culturing for 4 to 5 days, colonies formed were stained and the number of colonies was counted.

Binding study AUXB1 or CH^RC5 cells were plated at a density of 4×10^5 cells/well in 24-well plates (Falcon, no. 3047) and were cultured overnight at 37°C. The medium was replaced with 500 μl of Krebs-Ringer-phosphate buffer (KRP), which contained a constant amount of F-NCS at pH 7.4. The cells were then incubated at 37°C or 4°C for different time periods. At the end of the incubation period, the supernatant was discarded and the cells were washed three times with KRP to remove the unbound drug. For quantification of cell-associated F-NCS, cells were lysed in 1 ml of 50 m*M* Tris·HCl buffer, pH 8.5, containing 0.5% sodium dodecyl sulfate (SDS), and the fluorescence intensity of the lysates was measured by using the fluorescence spectrophotometer with excitation at 490 nm and emission at 520 nm.[1] Aliquots of the lysates were also used to quantify the protein content.

Efflux study AUXB1 and CH^RC5 cells were exposed to a constant amount of F-NCS for 80 min at 37°C or at 4°C in the same way as described above. Cells were rinsed three times with KRP and were incubated further at 37°C or at 4°C in drug-free growth medium. At various time periods, the medium was discarded, cells were washed once with KRP, and cell-associated drug and cellular protein were quantified after lysing the cells as described above.[1]

Antioxidant components Because of the known involvement of free radical(s) in the biological action of NCS,[10] we tested some radical scavengers. The content of total glutathione (GSH), nonprotein sulfhydryls (NPSH), GSH-*S*-transferase (GST), GSH peroxidase (GPX), Cu,Zn-containing superoxide dismutase (Cu,Zn-SOD) and Mn-containing SOD (Mn-SOD) in cells was measured according to the methods of Tietze,[13] Ellman,[14] Bjorn *et al.*,[15] Paglia and Valentine,[16] and Joenje *et al.*,[17] respectively.

Photolabeling of plasma membranes The plasma membranes were prepared according to the method described previously.[18] [^{3}H]Azidopine photolabeling was performed as described.[19] In brief, membrane fractions were incubated with 0.5 μM [^{3}H]azidopine for 15 min at room temperature in the presence and absence of 100 μM NPC, a putative competitor of [^{3}H]azidopine with respect to P-glycoprotein. After exposure to light at 366 nm for 20 min at 25°C using a fluorescence spectrophotometer with maximum slit opening (20 nm) (Hitachi Model 650-40), samples were solubilized in the buffer containing 4% SDS. Photolabeled membranes were then subjected to SDS-polyacrylamide gel electrophoresis (5.6%) containing 4.5 *M* urea at pH 7.4. Quantitation of radioactivity was made by using the cut gel corresponding to M_r 140,000–440,000, which contained P-glycoprotein, then it was dissolved in 31% H_2O_2 by heating, and the radioactivity was counted.

RESULTS

Cytotoxicity of NCS and NPC The cytotoxicity of NCS and NPC to both cell lines was examined by colony formation assay (Table II). The IC_{50} values (50% inhibitory concentration) of NCS for AUXB1 and CH^RC5 cells were 3.9 and 36 n*M*, respectively. On the other hand, the IC_{50} values of NPC for AUXB1 and CH^RC5 cells were 23 and 220 n*M*, respectively. CH^RC5 cells were about 10-fold more resistant than AUXB1 cells to both NCS and NPC.

Cell growth rate and antioxidant contents Doubling times of AUXB1 and CH^RC5 cells were 12 and 14 h, respectively. The rate of protein synthesis by the resistant cells at a semiconfluent state, as measured in terms of the incorporation of [^{3}H]-L-leucine, was about 85% of that of the parental cells (data not shown).

Some anticancer agents such as adriamycin, mitomycin C, and NCS are thought to be cytotoxic because of free radical formation via biological or chemical activation. Therefore, the enzyme activities of GST, GPX, and SOD and the contents of GSH and NPSH were examined in AUXB1 and CH^RC5 cells, because these enzymes and compounds are thought to reduce the cytotoxicity of alkylating agents and anthracyclines by acting as scavengers of free radicals.[20–22] On the other hand,

Table I. Antioxidant Levels in AUXB1 and CH^RC5 Cells

Compound/enzyme	AUXB1 cells	CH^RC5 cells
GSH	31.9±0.6	33.6±2.9
NPSH	38.4±2.5	33.2±2.1
GPX	28.6±2.0	26.2±1.6
GST	131.0±5.0	120.0±21.0
Cu,Zn-SOD	176.2±19.1	180.3±4.9
Mn-SOD	5.6±1.4	12.4±6.2

GSH and NPSH are expressed in nmol/mg protein, and enzymes in mU/mg protein. Each value is the mean±SD of four experiments.

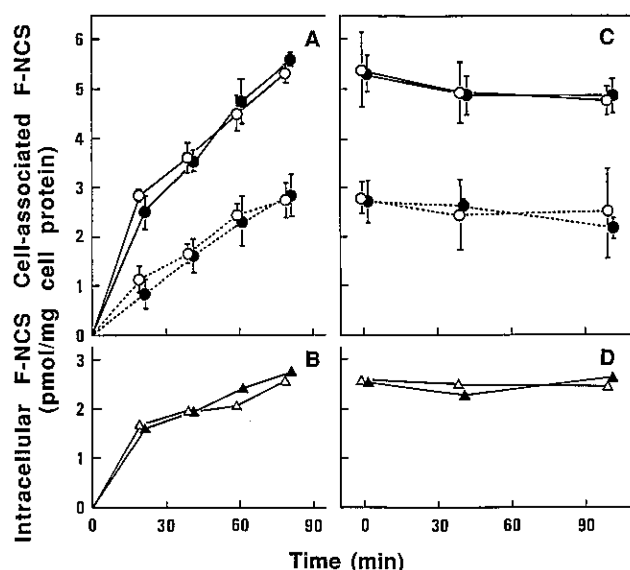

Fig. 1. Kinetics of binding (A and B) and efflux (C and D) of F-NCS (protein portion). (A) AUXB1 (○) and CH[R]C5 (●) cells were incubated with 20 μg/ml F-NCS in KRP at 37°C (——) or at 4°C (······). After incubation, washed cells were lysed and the amounts of cell-bound F-NCS and cellular protein were determined as described in the text. Each point is the mean value of three experiments ± SD (bars). (B) Intracellular accumulations of F-NCS in AUXB1 (△) and CH[R]C5 (▲) cells were calculated by subtracting the amounts of cell-associated F-NCS at 4°C from those at 37°C. (C) AUXB1 (○) and CH[R]C5 (●) cells were exposed to 20 μg/ml F-NCS for 80 min at 37°C or at 4°C and washed. Cells were further incubated at 37°C (——) or at 4°C (······) without drug. F-NCS and protein contents were determined after lysing the cells as described in the text. Each point is the mean value of three experiments ± SD (bars). (D) Intracellular retentions of F-NCS in AUXB1 (△) and CH[R]C5 (▲) cells were calculated in the same way as mentioned above.

NPC is activated by GSH or NPSH to generate the NPC radical.[10, 23] The results shown in Table I indicate, however, that the levels of these compounds and enzymes were not significantly elevated or decreased in the resistant cells.

Influx and efflux of F-NCS Sehested *et al.* reported that endocytosis was increased in a multidrug-resistant cell line compared with the sensitive parent.[24] However, there was little difference in binding and uptake of F-NCS to cells at 37°C or at 4°C between AUXB1 and CH[R]C5 cells (Fig. 1A), indicating the endocytotic uptake of the drug by these two cell lines was almost the same. For this calculation the amount of cell-associated F-NCS at 4°C was considered as the drug on the cell surface, and thus this value was subtracted from that at 37°C to get the value of the intracellular F-NCS (Fig. 1B). Furthermore, the intracellular F-NCS was retained well by both cell lines, and we did not find enhanced efflux of F-NCS from CH[R]C5 cells compared with that from AUXB1 cells (Fig. 1C and D).

Effect of P-glycoprotein inhibitors Because a calcium channel blocker, verapamil, and a biscoclaurine alkaloid, cepharanthine, are known to inhibit P-glycoprotein-dependent active efflux of anticancer drugs and to reverse multidrug resistance,[3, 4] we tested the effects of these compounds on the cytotoxicity of NCS to these two cell lines (Table II). The sensitivity of the resistant cell line to NCS was found to be increased up to 4 and 10 times in the presence of 20 μM verapamil and 27 μM cepharanthine, respectively.

Almost all cytotoxic functions of NCS are regarded as being due to NPC and the protein portion is believed to act only as a carrier/stabilizer of NPC. Therefore, we also examined the effects of verapamil and cepharanthine on the cytotoxicity of NPC (Table II). Both 20 μM

Table II. Effects of Verapamil and Cepharanthine on the Cytotoxicity of NCS and NPC

Drug	IC_{50} (nM)[a]		Resistance index[c]
	AUXB1 cells	CH[R]C5 cells	
NCS	3.9±1.4[b]	36.2±17.2[b]	9.4
NCS+verapamil	3.8	8.4	2.2
NCS+cepharanthine	3.5	3.6	1.0
NPC	19.9±11.4	184.3±67.5	11.2
NPC+verapamil	28.3	11.5	0.4
NPC+cepharanthine	7.6	12.4	1.6

a) Cells were incubated with each drug at various concentrations for 24 h at 37°C and colony numbers were counted. The IC_{50} values were obtained. Inhibition of colony formation was determined as described in the text. Concentrations of verapamil and cepharanthine were 20 μM and 27 μM, respectively.

b) IC_{50} values for NCS or NPC alone are the mean±SD for five experiments. The other values are the results of duplicate examinations.

c) Resistance index=(IC_{50} for CH[R]C5 cells)/(IC_{50} for AUXB1 cells).

Table III. Incorporation of [^{3}H]Azidopine into the Plasma Membranes of AUXB1 and CH^{R}C5 Cells in the Presence and Absence of NPC[a]

Cell line	NPC (μM)	Radioactivity[b] (dpm)	Suppression of incorporation of [^{3}H]azidopine (%)[c]
AUXB1	0	68.3	—
CH^{R}C5	0	4797.7	0
CH^{R}C5	100	2808.3	41

a) [^{3}H]Azidopine was used in this experiment at 0.5 μM.
b) Each value is the mean of duplicate experiments.
c) Suppression of incorporation (%) = {1−(radioactivity in the presence of NPC)/(radioactivity in the absence of NPC)} ×100.

verapamil and 27 μM cepharanthine increased the susceptibility of CH^{R}C5 cells to NPC about 9 fold, to the level of AUXB1 cells. On the other hand, the extracellular calcium concentration did not affect the cytotoxicity of either NCS or NPC (data not shown).

Furthermore, we examined the suppressing effect of NPC on the photolabeling of plasma membranes of CH^{R}C5 cells with [^{3}H]azidopine, which is known to react covalently with P-glycoprotein. In the presence of a 200-fold molar excess of NPC to [^{3}H]azidopine, the radiolabeling of plasma membranes of CH^{R}C5 cells was suppressed to 59% (Table III). Contrary to the case of CH^{R}C5 cells the radioactivity in the plasma membranes of AUXB1 cells was very low, i.e., 1.3% of that of CH^{R}C5 cells, which means that rather few target sites for [^{3}H]azidopine are present in the sensitive cells. In other words, most of the radioactivity seems to bind to P-glycoprotein, which is known to have M_r about 170,000.[3, 4]

DISCUSSION

The results of this study suggest that the active efflux of NPC of NCS by P-glycoprotein is one of the mechanisms of resistance to NCS in the multidrug-resistant cell line used. The results also indicate that NCS releases low-molecular-weight NPC in the cells, and NPC is then incorporated into a part of the P-glycoprotein-dependent efflux system.

Multidrug-resistant CH^{R}C5 cells had a slightly lower growth rate than the parental AUXB1 cells, although the difference was small. As we reported previously, NCS can kill the cells in a non-time-dependent manner.[1] Because of this rapid cytotoxic action, it is not likely that the small difference in cell growth rate is one of the main mechanisms of the difference in the sensitivity of the two cell lines to the agent.

NPC, a bicyclodiynene antibiotic, has a unique, labile nine-membered ring structure.[25] Edo *et al.* observed free radical generation from NPC in the presence of 2-mercaptoethanol,[23] and recently, the structure of the NPC radical and the mechanism of DNA scission by this radical in the presence of oxygen were proposed.[26, 27]

Therefore, we measured the contents of GSH and the activities of relevant enzymes and SOD (Table I), which are thought to scavenge the active oxygen species and have been reported to be associated with the expression of drug resistance in some cell lines.[21, 28–31] The sensitivity of the cells to NCS could be reduced by depletion of cellular GSH.[32, 33] However, CH^{R}C5 cells contained the same levels of GSH and NPSH as AUXB1 cells (Table I). Although the difference was not statistically significant ($P>0.10$), Mn-SOD in the resistant cells was about two times more active than that in AUXB1 cells. Mitochondria, however, are not considered to be the target for NCS. The activities of the other antioxidant enzymes measured in the resistant cell line were almost the same as those in the sensitive parental cell line (Table I). Therefore, these compounds and enzymes may not play an important role in the differential sensitivity to NCS in the cell lines used.

The results shown in Fig. 1 indicate that at least the protein portion of the drug was incorporated and retained in the two cell lines to the same degree. In addition, the association kinetics of fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (F-BSA) and FITC-labeled SMANCS (styrene-maleic acid copolymer-conjugated NCS) (F-SMANCS) in CH^{R}C5 cells were not different from those in AUXB1 cells (ref. 1 and unpublished data), which indicates that the transmembrane protein transport activities, i.e., endocytosis and exocytosis, were not different in the multidrug-resistant and parental cells.

Many chemical agents have been shown to reverse multidrug resistance in *in vitro* studies. For instance, verapamil and cepharanthine were reported to act on P-glycoprotein and to interfere with the efflux of several anticancer agents from cells.[18, 34] Therefore the results shown in Table II suggest that the resistance of CH^{R}C5 cells to NCS or NPC may be attributable to a P-glycoprotein-dependent mechanism. Verapamil affects the influx of calcium and may influence various biochemical functions of the cells. Calcium depletion by the use of 0.125 mM EDTA in calcium- and magnesium-free Hanks' balanced salt solution, at pH 7.4, which was used for drug treatment, however, did not modulate the cytotoxicity of NCS to the two cell lines used (data not shown). Furthermore, cepharanthine, which is known to act as a membrane stabilizer, has not been reported to inhibit calcium influx. The potentiating effect of this agent on NPC in AUXB1 cells (Table II) may be ex-

plained by the fact that diffusion out of the membrane may be suppressed due to its stabilizing effect on the membrane. Therefore, the reversal of the resistance to NCS and NPC by these agents may not be a result of the decrease in intracellular calcium concentration.

In this context, we carried out one more experiment using [^{3}H]azidopine, which is known to bind P-glycoprotein selectively,[19] to see whether NPC and [^{3}H]-azidopine compete with each other in their binding to P-glycoprotein. Namely, a 200-fold excess NPC had a suppressive effect on the labeling of P-glycoprotein by [^{3}H]azidopine. This seems to suggest that NPC binds to the same or a closely related site on P-glycoprotein. As expected, the sensitive cell line AUXB1 showed only 1.3% as much binding of [^{3}H]azidopine due to the small amount of P-glycoprotein (Table III).

Keizer Joenje reported that the intracellular pH of multidrug-resistant human lung tumor cells was higher than that of the parental cells.[35] NPC is reported to be unstable in the high pH environment,[36] but it is unreasonable to assume that the resistance of CH^RC5 cells to NCS or NPC is due to the higher intracellular pH. Our preliminary experiment using F-NCS to measure the intracellular pH[9] of both cell lines showed almost no difference (data not shown). Furthermore, phorbol myristate acetate, which is a potent protein kinase C activator and also acts on Na^+,K^+-ATPase to increase intracellular pH,[37] did not affect the cytotoxicity of NCS in the two cell lines used (data not shown). Thus, the intracellular pH can not explain the present observation of different sensitivity in the two cell lines.

All these results suggest that the efflux of NPC by P-glycoprotein is one of the main mechanisms of the resistance of CH^RC5 cells to NCS or NPC. NPC is an amphipathic and cationic molecule (M_r 659); these properties are consistent with the P-glycoprotein-dependent efflux mechanism as reported.[3,4] Therefore, NPC liberated from NCS in the cytosol may be excreted via P-glycoprotein in multidrug-resistant cells. The direct quantification of intracellular accumulation of NPC in both cell lines was tried, but the very labile character of NPC and the minute quantity present (pmol/mg cell protein) made accurate determination very difficult.

ACKNOWLEDGMENTS

The authors thank Prof. Victor Ling for providing the two cell lines, AUXB1 and CH^RC5. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture.

(Received September 19, 1990/Accepted December 28, 1990)

REFERENCES

1) Miyamoto, Y., Oda, T. and Maeda, H. Comparison of the cytotoxic effects of the high- and low-molecular-weight anticancer agents on multidrug-resistant Chinese hamster ovary cells *in vitro*. *Cancer Res.*, **50**, 1571–1575 (1990).
2) Oda, T., Sato, F., Yamamoto, H., Akagi, M. and Maeda, H. Cytotoxicity of smancs in comparison with other anticancer agents against various cells in culture. *Anticancer Res.*, **9**, 261–266 (1989).
3) Bradley, G., Juranka, P. F. and Ling, V. Mechanism of multidrug resistance. *Biochim. Biophys. Acta*, **948**, 87–128 (1988).
4) Tsuruo, T. Mechanisms of multidrug resistance and implications for therapy. *Jpn. J. Cancer Res.*, **79**, 285–296 (1988).
5) Ling, V. and Thompson, L. H. Reduced permeability in CHO cells as a mechanism of resistance to colchicine. *J. Cell Physiol.*, **83**, 103–116 (1974).
6) Maeda, H., Aikawa, S. and Yamashita, A. Subcellular fate of protein antibiotic neocarzinostatin in culture of a lymphoid cell line from Burkitt's lymphoma. *Cancer Res.*, **35**, 554–559 (1975).
7) Takeshita, J., Maeda, H. and Koike, K. Subcellular action of neocarzinostatin. *J. Biochem.*, **88**, 1071–1080 (1980).
8) Oda, T. and Maeda, H. Binding to and internalization by cultured cells of neocarzinostatin and enhancement of its actions by conjugation with lipophilic styrene-maleic acid copolymer. *Cancer Res.*, **47**, 3206–3211 (1987).
9) Oda, T., Sato, F. and Maeda, H. Facilitated internalization of neocarzinostatin and its lipophilic polymer conjugate, SMANCS, into cytosol in acidic pH. *J. Natl. Cancer Inst.*, **79**, 1205–1211 (1987).
10) Goldberg, I. H. Free radical mechanisms in neocarzinostatin-induced DNA damage. *Free Radical Biol. Med.*, **3**, 41–54 (1987).
11) Napier, M. A., Goldberg, I. H., Hensens, O. D., Dewey, R. S., Liesch, J. M. and Albers-Schönberg, G. Neocarzinostatin chromophore: presence of a cyclic carbonate subunit and its modification in the structure of other biologically active forms. *Biochem. Biophys. Res. Commun.*, **100**, 1703–1712 (1981).
12) Maeda, H., Ishida, N., Kawauchi, H. and Tsuzimura, K. Reaction of fluorescein isothiocyanate with proteins and amino acids. I. Covalent and non-covalent binding. *J. Biochem.*, **65**, 777–783 (1969).
13) Tietze, F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.*, **27**, 502–522 (1969).
14) Ellman, G. E. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, **82**, 70–77 (1959).

15) Björn, D., Martinsson, T., Mannervik, B., Jensson, H. and Levan, G. Characterization of multidrug resistance in SEWA mouse tumor cells: increased glutathione transferase activity and reversal of resistance with verapamil. *Anticancer Res.*, **7**, 65–70 (1987).
16) Paglia, D. E. and Valentine, W. N. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.*, **70**, 158–169 (1967).
17) Joenje, H., Gille, J. J. P., Oosta, A. B. and Van der Valk, P. Some characteristics of hyperoxia-adapted HeLa cells. A tissue culture model for cellular oxygen tolerance. *Lab. Invest.*, **52**, 420–428 (1985).
18) Yusa, K. and Tsuruo, T. Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res.*, **49**, 5002–5006 (1989).
19) Safa, A. R., Glover, C. J., Sewell, J. L., Meyers, M. B., Biedler, J. L. and Felsted, R. L. Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. *J. Biol. Chem.*, **262**, 7884–7888 (1987).
20) Hansson, J., Edgren, M., Ehrsson, H., Ringborg, U. and Nilsson, B. Effect of D,L-buthionine-*S,R*-sulfoximine on cytotoxicity and DNA cross-linking induced by bifunctional DNA-reactive cytostatic drugs in human melanoma cells. *Cancer Res.*, **48**, 19–26 (1988).
21) Lutzky, J., Astor, M. B., Taub, R. N., Baker, M. A., Bhalla, K., Gervasoni, J. E., Jr., Rosado, M., Stewart, V., Krishna, S. and Hindenburg, A. A. Role of glutathione and dependent enzymes in anthracycline-resistant HL60/AR cells. *Cancer Res.*, **49**, 4120–4125 (1989).
22) Doroshow, J. H. Role of hydrogen peroxide and hydroxyl radical formation in the killing of Ehrlich tumor cells by anticancer quinones. *Proc. Natl. Acad. Sci. USA*, **83**, 4514–4518 (1986).
23) Edo, K., Iseki, S., Ishida, N., Horie, T., Kusano, G. and Nozoe, S. An electron spin resonance study of a spin adduct of the non-protein component (NPC) of neocarzinostatin. *J. Antibiot.*, **33**, 1586–1589 (1980).
24) Sehested, M., Skovsgaard, T., van Deurs, B. and Winther-Nielsen, H. Increase in nonspecific adsorptive endocytosis in anthracycline- and vinca alkaloid-resistant Ehrlich ascites tumor cell lines. *J. Natl. Cancer Inst.*, **78**, 171–177 (1987).
25) Edo, K., Mizugaki, M., Koide, Y., Seto, H., Furihata, K., Otake, N. and Ishida, N. The structure of neocarzinostatin chromophore possessing a novel bicyclo-(7,3,O)-dodecadiyne system. *Tetrahedron Lett.*, **26**, 331–334 (1985).
26) Myers, A. G., Proteau, P. J. and Handel, T. M. Stereochemical assignment of neocarzinostatin chromophore. Structures of neocarzinostatin chromophore-methyl thioglycolate adducts. *J. Am. Chem. Soc.*, **110**, 7212–7214 (1988).
27) Chin, D-H., Zeng, C-H., Costello, C. E. and Goldberg, I. H. Sites in diyne-ene bicyclic core of neocarzinostatin chromophore responsible for hydrogen abstraction from DNA. *Biochemistry*, **27**, 8106–8114 (1988).
28) Mimnaugh, E. G., Dusre, L., Atwell, J. and Myers, C. E. Differential oxygen radical susceptibility of adriamycin-sensitive and -resistant MCF-7 human breast tumor cells. *Cancer Res.*, **49**, 8–15 (1989).
29) Sinha, B. K., Mimnaugh, E. G., Rajagopalan, S. and Myers, C. E. Adriamycin activation and oxygen free radical formation in human breast tumor cells: protective role of glutathione peroxidase in adriamycin resistance. *Cancer Res.*, **49**, 3844–3848 (1989).
30) Dulhanty, A. M., Li, M. and Whitmore, G. F. Isolation of Chinese hamster ovary cell mutants deficient in excision repair and mitomycin C bioactivation. *Cancer Res.*, **49**, 117–122 (1989).
31) Deffie, A. M., Alam, T., Seneviratne, C., Beenken, S. W., Batra, J. K., Shea, T. C., Henner, W. D. and Goldenberg, G. J. Multifactorial resistance to adriamycin: relationship of DNA repair, glutathione transferase activity, drug efflux, and P-glycoprotein in cloned cell lines of adriamycin-sensitive and -resistant P388 leukemia. *Cancer Res.*, **48**, 3595–3602 (1988).
32) DeGraff, W. G., Russo, A. and Mitchell, J. B. Glutathione depletion greatly reduces neocarzinostatin cytotoxicity in Chinese hamster V79 cells. *J. Biol. Chem.*, **260**, 8312–8315 (1985).
33) DeGraff, W. G. and Mitchell, J. B. Glutathione dependence of neocarzinostatin cytotoxicity and mutagenicity in Chinese hamster V-79 cells. *Cancer Res.*, **45**, 4760–4762 (1985).
34) Shiraishi, N., Akiyama, S., Nakagawa, M., Kobayashi, M. and Kuwano, M. Effect of bisbenzylisoquinoline (biscoclaurine) alkaloids on multidrug resistance in KB human cancer cells. *Cancer Res.*, **47**, 2413–2416 (1987).
35) Keizer, H. G. and Joenje, H. Increased cytosolic pH in multidrug-resistant human lung tumor cells: effect of verapamil. *J. Natl. Cancer Inst.*, **81**, 706–709 (1989).
36) Kappen, L. S. and Goldberg, I. H. Stabilization of neocarzinostatin nonprotein chromophore activity by interaction with apoprotein and with HeLa cells. *Biochemistry*, **19**, 4786–4790 (1980).
37) Moolenaar, W. H., Tertoolen, L. G. and deLaat, S. W. Phorbol ester and diacylglycerol mimic growth factors in raising cytoplasmic pH. *Nature*, **312**, 371–374 (1984).