

Circumvention of multidrug resistance in neoplastic cells through scavenger receptor mediated drug delivery

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Abstract A conjugate of the antineoplastic drug daunomycin (DNM) with maleylated bovine serum albumin (MBSA-DNM) was taken up with high efficiency by a multidrug resistant variant, JD100, of the murine-macrophage tumour cell line, J774A.1, through the scavenger receptors resulting in cessation of DNA synthesis. In contrast, free DNM at similar concentrations did not affect the incorporation of [³H]thymidine by these cells. These results suggest that receptor-mediated intracellular delivery of antineoplastic drugs could be a viable and new approach for overcoming the problem of multidrug resistance in chemotherapy of neoplastic diseases.

Key words: Multidrug resistance; Scavenger receptor; Endocytosis; Drug targeting

1. Introduction

Resistance to a wide variety of structurally and functionally dissimilar cytotoxic drugs, termed multidrug resistance (MDR), is a major obstacle to success in chemotherapy of human cancers. MDR in human cancer appears to be multifactorial. In a large number of cancer cell lines and some human hematopoietic malignancies, MDR is associated with the overproduction of a membrane protein, P-glycoprotein (Pgp). Pgp is an ATP-dependent pump of broad specificity which mediates efflux of many amphipathic lipophilic drugs, and thus prevents accumulation of drug in sufficient concentration effective in hitting the intracellular target sites (reviewed in [1,2]). Mechanisms other than Pgp-mediated transport also appear to reduce intracellular drug levels in cancer cells, for which sufficient molecular details are not yet available [3,4]. The mainstay of current strategy for overcoming MDR in cancer chemotherapy is combination regimens with agents that block the action or expression of Pgp [5–8]. However, alternative strategies to overcome multidrug resistance need to be developed since such blockade of Pgp function in normal cells could exert deleterious effects, and, additionally, all clinical manifestations of MDR are unlikely to be mediated by Pgp.

Since Pgp mediates efflux of drugs in the plasma membrane, it is conceivable that this barrier can be circumvented by the use of liposome-encapsulated drugs [9–11] and receptor-mediated drug delivery systems (reviewed in [12]). We have previ-

ously shown that cytotoxicity of daunomycin (DNM) against human histiocytic lymphoma U937 in vitro [13], and murine J774A.1 tumour cells in vitro and in vivo [14,15], can be markedly enhanced by conjugation of the drug with maleylated bovine serum albumin (MBSA) which is recognized by scavenger receptors expressed primarily on cells of macrophage lineage [16–18]. To overcome the Pgp efflux pump and other putative permeability barriers, in the present study we have utilized the approach of scavenger-receptor mediated delivery of macromolecular drug conjugates to multidrug resistant cells. We show that the DNM-conjugate of MBSA is recognised by scavenger receptors and causes cessation of DNA synthesis in a DNM-resistant subline, JD100, derived from murine macrophage tumour cell line J774A.1. To our knowledge, this is the first report demonstrating the efficacy of receptor-mediated drug delivery approach to restore DNM sensitivity in DNM-resistant cells exhibiting multidrug resistance.

2. Materials and methods

2.1. Materials

Tissue culture supplies were from Grand Island Biological Co. (Grand Island, NY). Tissue culture media used were as follows: Medium A consisted of DMEM with 10% fetal calf serum (FCS), 2 mM L-glutamine, 0.37% sodium bicarbonate, and 50 µg/ml gentamycin; Medium B is medium A containing 100 ng/ml of DNM; Medium C is medium A without bicarbonate and FCS but containing 1 mg/ml bovine serum albumin (BSA); Medium D is medium C with bicarbonate. DNM, BSA, polyguanylic acid, and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO). [³H]Thymidine and Na¹²⁵I were purchased from Amersham International (Amersham, UK). Other reagents used were of analytical grade.

2.2. Cells

J774A.1, a mouse tumour cell line of monocyte-macrophage origin (American Type Culture Collection) was cultured in medium A at 37°C in a 5% CO₂–95% air atmosphere.

2.3. Development of drug resistant cells

A drug-resistant subline of J774A.1 cells was developed by continuous exposure of these cells to stepwise increasing concentrations of DNM from 25–100 ng/ml over the course of about a year as described [19]. These cells, named JD 100, were maintained in medium A containing 20% FCS in presence of DNM (100 ng/ml). The DNM-resistant phenotype expressed by these cells was stable for at least six months during the course of this study.

2.4. Preparation of drug conjugate

BSA was maleylated at pH 8.0 by using maleic anhydride as previously described [14]. To prepare daunomycin drug conjugate, 3 mg of MBSA was mixed with 400 µg of DNM in 1 ml phosphate-buffered saline (PBS) followed by dropwise addition of glutaraldehyde (0.1%) to a final concentration of 0.01% of glutaraldehyde. The contents were stirred for 15 min at room temperature. The reaction was stopped by adding 50 µl of 1 M lysine followed by separation of drug conjugate from low molecular weight components by Sephadex G-50 gel filtration. Void volume fractions containing the MBSA–DNM conjugate

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Abbreviations: MDR, multidrug resistance; MBSA, maleylated bovine serum albumin; DNM, daunomycin; Pgp, P-glycoprotein; PBS, phosphate-buffered saline; DXR, doxorubicin; Mtx, methotrexate; Vin, vincristine; 5-Fu, 5-fluorouracil; Mit, mitomycin C; FCS, fetal calf serum; TCA, trichloroacetic acid.

were pooled and subjected to extensive dialysis against PBS. DNM content of the conjugate was determined by measuring the absorbance at 495 nm [20]. MBSA showed no absorbance at 495 nm. Protein was measured by using bicinchoninic acid (BCA) reagent [21]. The drug conjugate contained 3–4 moles of DNM per mole of MBSA.

2.5. Radioiodination of MBSA–DNM

MBSA–DNM conjugate was radioiodinated with Na¹²⁵I by the iodine monochloride method as previously described [14,22]. More than 99% of the radioactivity associated with the drug conjugate was trichloroacetic acid (TCA)-precipitable. The specific activity of the radiolabeled drug conjugate was approximately 120 cpm/ng of protein.

2.6. Binding of [¹²⁵I]MBSA–DNM by JD 100 and J774A.1 cells at 4°C

J774A.1 and JD100 cells (5×10^5 cells) in 24-well tissue culture clusters were incubated in 1 ml of medium A or medium B, respectively, at 37°C. After 24 h, monolayers were washed twice with 1 ml of ice cold FCS free medium A. These cells were incubated at 4°C in 1 ml of medium C containing 6 µg/ml of [¹²⁵I]MBSA–DNM in the presence and absence of competing compounds, i.e. MBSA (300 µg/ml), polyguanylic acid (300 µg/ml), and DNM (5 µg/ml). After 2 h, cells were washed three times with ice-cold PBS containing 1 mg/ml of BSA and once with PBS. The cells were dissolved in 1 ml of 0.1 N NaOH, and the amount of radioactivity associated with the cells was determined [14]. Cellular protein was estimated using an aliquot of the cell lysate by BCA method [21]. Results were expressed as ng of [¹²⁵I]MBSA–DNM bound per mg of cellular protein.

2.7. Assay of uptake and degradation of [¹²⁵I]MBSA–DNM

JD100 and J774A.1 monolayer cells in 24-well tissue culture plates as described earlier were washed twice with 1 ml of FCS free medium A. To each well was added 1 ml of medium D containing 6 µg/ml of [¹²⁵I]MBSA–DNM. Where indicated, the competing compounds, MBSA (300 µg/ml), polyguanylic acid (300 µg/ml), and DNM (5 µg/ml) were added to the wells and contents incubated at 37°C in 5% CO₂–95% air atmosphere. After 5 h, the amount of TCA soluble (noniodide) radioactivity released in the medium was measured as described [14,22]. Monolayer cells were washed three times with ice cold PBS containing 1 mg/ml of BSA and once with PBS. The cells were lysed in 1 ml of 0.1 N NaOH and the cell-associated cellular radioactivity and protein content were estimated as described above. Results were expressed as ng of [¹²⁵I]MBSA–DNM degraded per mg of cellular protein.

2.8. Cytotoxicity assay in vitro

The cytotoxicity of the drug conjugate was assayed by the [³H]thymidine uptake assay. J774A.1 and JD100 monolayer (1×10^5 cells) in 24-well tissue culture clusters were washed twice with 1 ml of medium A and the cells were incubated in 1 ml of medium A containing indicated concentrations of DNM either in free or conjugated form, for 60 min (Fig. 2), or indicated times (Fig. 3), at 37°C in an incubator containing 5% CO₂. Subsequently, the cells were washed thrice with 1 ml of medium A and incubated in 1 ml of drug free medium at 37°C. After 20 h the cells were incubated with 1 ml of medium A containing [³H]thymidine (2 µCi/ml) at 37°C for 3 h. The cells were then washed four times with 1 ml of medium A to remove the unincorporated radioactivity. Cells were then lysed in 0.5 ml of 0.1 N NaOH and the amount of radioactivity associated with the cells was determined. The results are expressed as percentage of [³H]thymidine incorporated of the control cells.

3. Results and discussion

A DNM-resistant subline, referred to as JD100, was generated by stepwise exposure of murine macrophage J774A.1 tumor cells to increasing concentrations of DNM as described in section 2. Table 1 shows the concentrations of different drugs required to cause 50% inhibition of [³H]thymidine incorporation by the parental J774A.1 and the drug-resistant JD100 cells. JD100 cells were 15-fold more resistant to DNM compared to J774A.1 cells and also showed cross-resistance to doxorubicin (DXR, 32-fold), methotrexate (Mtx, 60-fold), vincristine (Vin,

Table 1

Inhibition of [³H]thymidine incorporation by J774A.1 and JD100 cells by different drugs

Drugs	IC ₅₀ (µM)		Fold resistance
	J774A.1	JD100	
Daunomycin	0.05	0.75	15
Doxorubicin	0.025	0.81	32.4
Methotrexate	0.5	30	60
Vincristine	0.007	0.11	16
5-Fluorouracil	0.77	4.3	5.6
Mitomycin C	0.3	1	3

J774A.1 and JD100 cells (1×10^5) were treated with different concentrations of the indicated drugs for 20 h, and subsequently exposed to [³H]thymidine (2 µCi/ml) for 3 h as described in section 2. [³H]thymidine incorporation by untreated cells was taken as 100%. Concentration of respective drugs required for 50% inhibition of [³H]thymidine incorporation (IC₅₀) by the respective cells are tabulated here and the results are expressed as the average of three independent determinations.

16-fold), 5-fluorouracil (5-Fu, 6-fold), and mitomycin C (Mit C, 3-fold) suggesting that the JD100 cells had acquired the MDR phenotype.

To further characterise the MDR phenotype in JD100 cells, we determined the cytotoxic activity of above mentioned drugs on JD100 cells in the presence and absence of 20 µM verapamil, an inhibitor of Pgp mediated drug efflux [23]. As shown in Table 2, DNM, DXR, Vin, and Mit C exerted substantial cytotoxic effect on JD100 cells as measured by [³H]thymidine incorporation in the presence of 20 µM verapamil, suggesting that resistance to these drugs in JD100 cells was mediated by Pgp. Drugs known to be insensitive to Pgp-mediated efflux, viz., Mtx and 5-Fu, did not show significant cytotoxic activity even in presence of verapamil. These data suggest that the MDR phenotype expressed by the JD100 cells is of a complex nature resembling that often found in clinical situations [5,24].

To determine whether the acquisition of the MDR phenotype by JD100 cells had affected the functional properties of the scavenger receptors on JD100 cells, we compared the ability of J774A.1 and JD100 cells to bind and degrade [¹²⁵I]MBSA–DNM. In the absence of any competing compound, approximately 300 ng of MBSA–DNM per mg of cellular protein bound to both the cell types when incubated in presence of 6 µg/ml of [¹²⁵I]MBSA–DNM at 4°C for 2 h (Fig. 1a). The binding of MBSA–DNM by both the cell types was inhibited

Table 2

Reversal of resistance to different drugs in JD100 cells by verapamil

Drugs	% of [³ H]thymidine incorporation	
	– Verapamil	+ Verapamil
No addition	100	84
0.05 µM Daunomycin	102	36
0.025 µM Doxorubicin	98	46
0.007 µM Vincristine	76	8.7
0.3 µM Mitomycin C	81	49
0.5 µM Methotrexate	95	87
0.77 µM 5-Fluorouracil	101	80

Cytotoxic activity of the indicated drugs was determined by measuring the amount of [³H]thymidine incorporated in the presence and absence of verapamil (20 µM) as described under Table 1. The drugs were used at IC₅₀ values for the parental J774A.1 cells as in Table 1. [³H]thymidine incorporation by untreated control cells was taken as 100% and the results are expressed as the average of three independent experiments.

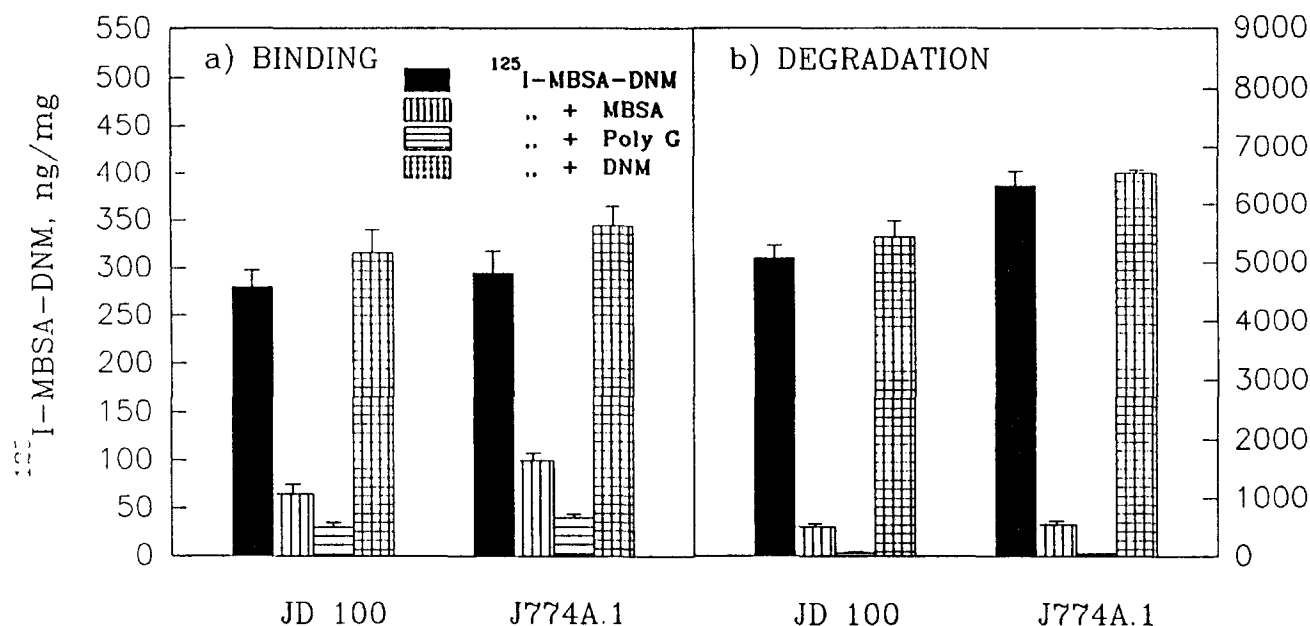


Fig. 1. Binding and degradation of [^{125}I]MBSA-DNM by JD100 and J774A.1 cells. (a) Binding of [^{125}I]MBSA-DNM (6 $\mu\text{g}/\text{ml}$) in absence or presence of MBSA (300 $\mu\text{g}/\text{ml}$), polyguanylic acid (300 $\mu\text{g}/\text{ml}$) and DNM (5 $\mu\text{g}/\text{ml}$) to the cells at 4°C was carried out as described in section 2. Results are expressed as ng of [^{125}I]MBSA-DNM bound per mg of cellular protein. (b) Degradation of [^{125}I]MBSA-DNM (6 $\mu\text{g}/\text{ml}$) alone or in the presence of the competing compounds as above was carried out as described in section 2. Results are expressed as ng of [^{125}I]MBSA-DNM degraded per mg of cellular protein. All values are corrected for the small amount of TCA-soluble ^{125}I radioactivity that was present in the medium incubated with [^{125}I]MBSA-DNM in the absence of cells, and represent average of triplicate incubations \pm S.D.

(>80%) in presence of known ligands of the scavenger receptor, viz., MBSA and polyguanylic acid, but not by free DNM indicating that the recognition of the drug-conjugate by both the cell lines was mediated through the scavenger receptor. In both the cell types, binding of the drug conjugate was followed by its rapid internalization and subsequent degradation. When incubated with 6 $\mu\text{g}/\text{ml}$ of [^{125}I]MBSA-DNM at 37°C for 5 min, J774A.1, and JD100 cells degraded approximately 6365 and 5071 ng of MBSA-DNM per mg of cellular protein, respectively; the degradation of MBSA-DNM was competed by MBSA and polyguanylic acid but not by free DNM in both the cell types (Fig. 1b). These data indicate that the drug resistant JD100 cells possessed functionally active scavenger receptors capable of efficient internalization of the drug conjugate at 37°C which is followed by the proteolytic degradation of the MBSA moiety.

To compare the cytotoxic efficacy of free and conjugated DNM on the sensitive J774A.1 and resistant JD100 cells, we incubated these cells in presence of the indicated concentrations of free and conjugated DNM at 37°C for 1 h, and measured the amount of [^3H]thymidine incorporated by these cells in drug-free medium for 3 h (Fig. 2). The data presented in Fig. 2a shown the dose dependent inhibition of [^3H]thymidine incorporation brought about by exposure of the drug sensitive J774A.1 cells to free DNM. There was 70% inhibition of [^3H]thymidine incorporation at 0.3 μM DNM compared to the untreated control, whereas free DNM under the same conditions did not affect [^3H]thymidine incorporation in the drug resistant JD100 cells. In contrast, treatment with conjugated DNM (0.3 μM) could inhibit [^3H]thymidine incorporation in drug resistant JD100 cells by 50% (Fig. 2b).

Fig. 3 illustrates the effects of free and conjugated DNM on

[^3H]thymidine incorporation by JD100 cells as a function of time of exposure to the drug. When JD100 cells were incubated with 0.3 μM DNM in the free form, the incorporation of [^3H]thymidine by JD100 cells was same as in untreated control cells over the experimental period of 240 minutes confirming the drug resistant phenotype expressed by the JD100 cells. In contrast, exposure of JD100 cells to DNM (0.3 μM) in the conjugated form resulted in 50% inhibition of [^3H]thymidine incorporation at 120 min.

The data presented in Figs. 2 and 3 indicate that the DNM-resistance phenotype displayed by the multidrug resistant

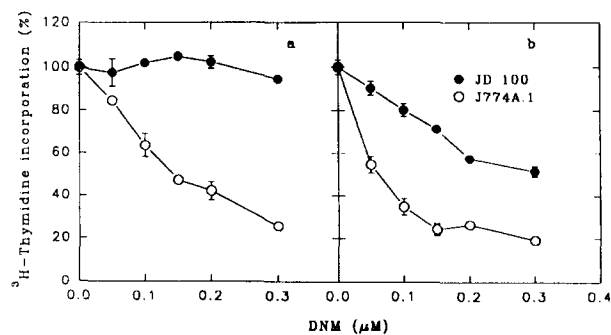


Fig. 2. Cytotoxic activity of MBSA-DNM and DNM on J774A.1 and JD100 cells at 37°C . J774A.1 and JD100 were treated at the given concentrations of DNM in free (a) or conjugated form (b) for 1 h as described in section 2. Subsequently, the cells were washed thrice with 1 ml of medium A, and in 1 ml of drug-free medium at 37°C . After 20 h, the cells were pulsed with 1 ml of [^3H]thymidine (2 $\mu\text{Ci}/\text{ml}$) containing medium A for 3 h at 37°C , and the amount of radioactivity associated with the cells were determined as in section 2. The results are expressed as percentage of [^3H]thymidine incorporation by the untreated cells (average of three independent experiments \pm S.D.).

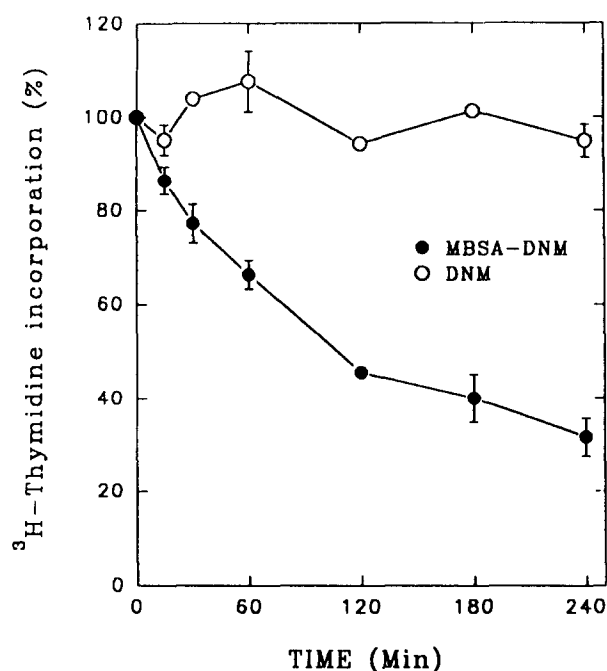


Fig. 3. Time course of killing of JD100 cells by MBSA-DNM or DNM at 37°C. Experiments were carried out as described under Materials and methods. JD100 cells were incubated with 0.3 μ M of DNM either in free or conjugated form for the indicated time periods, washed, and incubated in drug-free medium for 20 h. The cells were then pulsed with [3 H]thymidine (2 μ Ci/ml) in drug-free medium for 3 h, washed and the radioactivity associated with the cells was determined. Cell-associated radioactivity in untreated control cells was taken as 100% and the results were expressed as an average of percentage incorporation of three independent experiments \pm S.D.

JD100 cells could be largely overcome when the drug was presented as MBSA-DNM conjugate. In view of our previously reported results showing many fold higher intracellular concentrations achieved when scavenger receptor bearing cells are presented with drugs conjugated with MBSA [13,25], it is likely that the DNM-resistant JD100 cells become susceptible to drug because an inhibitory intracellular concentration of DNM could be achieved through scavenger receptor mediated uptake of MBSA-DNM. Alternatively, the receptor-mediated uptake of the drug conjugate may deliver the drug to an intracellular compartment which is not accessible to the Pgp-mediated efflux pump. It is also conceivable that the intralysosomal release of the drug through the receptor-mediated modality might provide a more effective access to the intracellular target of DNM action. Further studies are required to clarify the mechanism of the superior efficacy of MBSA-DNM in circumventing drug resistance.

The idea of utilizing ligands of surface receptors as carriers of drugs, both to target them to specific cells and generate optimal intracellular concentration has been exploited earlier by our laboratory [13–15,25] and by others (reviewed in [12]). However, no attempts have yet been reported to apply the aforementioned approach to overcome the problem of MDR. Our studies thus demonstrate the general principle that the process of receptor-mediated endocytosis of drug conjugates

could be a viable approach for killing drug resistant neoplastic cells if the receptor systems exclusive for such cells are known. The present investigation suggests that this approach could permit use of the same drug, albeit in the conjugated form, in contrast to the use of immunotoxins. The current strategies for circumventing MDR, viz., use of chemosensitizers, liposomes, immunotoxins, or antibodies have serious drawbacks while the current approach of receptor-mediated intracellular delivery of anticancer drugs for overcoming multidrug resistance shows promise requiring further studies.

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References

- [1] Endicott, J.A. and Ling, V. (1989) *Annu. Rev. Biochem.* 58, 127–171.
- [2] Gottesman, M.M. and Pastan, I. (1993) *Annu. Rev. Biochem.* 62, 385–427.
- [3] Eijdens, W.H.M., Borst, P., Jongsma, A.P.M., De Jong, S., De Vries, E.G.E., Groenigen, M.V., Versantvoort, C.H.M., Nieuwint, A.W.M. and Baas, M. (1992) *Proc. Natl. Acad. Sci. USA* 89, 3498–3502.
- [4] Marquardt, D. and Center, M.S. (1992) *Cancer Res.* 52, 3157–3163.
- [5] Ford, J.M., and Hait, W.N. (1990) *Pharmacol. Rev.* 42, 155–199.
- [6] Heike, Y., Okumura, K. and Tsuruo, T. (1992) *Japan J. Cancer Res.* 83, 366–372.
- [7] Thierry, A.R., Rahman, A. and Dritschilo, A. (1993) *Biochem. Biophys. Res. Commun.* 190, 952–960.
- [8] Verweij, J., Herweijer, H., Oosterom, R., Vander Burg, M.E.L., Plantin, A.S.T., Seynaeve, C., Stoter, G. and Nooter, K. (1991) *Br. J. Cancer* 64, 361–364.
- [9] Warren, L., Jardillier, J.C., Malaska, A. and Akeli, M.G. (1992) *Cancer Res.* 52, 3241–3245.
- [10] Mickish, G.H., Rahman, A., Pastan, I. and Gottesman, M.M. (1992) *J. Natl. Cancer Inst.* 84, 804–805.
- [11] Mayhew, E.G., Lasic, D., Babbar, S. and Martin, F.J. (1992) *Int. J. Cancer* 51, 302–309.
- [12] Basu, S.K. (1990) *Biochem. Pharmacol.* 40, 1941–1946.
- [13] Basu, S., Mukhopadhyay, B., Basu, S.K. and Mukhopadhyay, A. (1994) *FEBS Lett.* 342, 249–254.
- [14] Mukhopadhyay, A., Mukhopadhyay, B., Srivastava, R.K. and Basu, S.K. (1992) *Biochem. J.* 284, 237–241.
- [15] Mukhopadhyay, B., Mukhopadhyay, A. and Basu, S.K. (1993) *Biochem. Pharmacol.* 46, 919–924.
- [16] Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S. (1979) *Proc. Natl. Acad. Sci. USA* 76, 333–337.
- [17] Brown, M.S., Basu, S.K., Falck, J.R., Ho, Y.K. and Goldstein, J.L. (1980) *J. Supramol. Struct.* 13, 67–81.
- [18] Pitas, R.E., Boyles, J., Mahley, R.W. and Bissel, D.M. (1985) *J. Cell Biol.* 100, 103–117.
- [19] Roy, S.N. and Horwitz, S.B. (1985) *Cancer Res.* 45, 3856–3863.
- [20] Ghose, T.I., Blair, A.H. and Kulkarni, P.N. (1983) *Methods Enzymol.* 93, 280–333.
- [21] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Greke N.M., Olson, B.J. and Klinth, D.C. (1985) *Anal. Biochem.* 150, 76–85.
- [22] Goldstein, J.L., Basu, S.K. and Brown, M.S. (1983) *Methods Enzymol.* 98, 241–260.
- [23] Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. (1981) *Cancer Res.* 41, 1967–1972.
- [24] Simon, S.M. and Schindler, M.O. (1994) *Proc. Natl. Acad. Sci. USA* 91, 3497–3504.
- [25] Mukhopadhyay, A., Chaudhury, G., Arora, S.K., Sehgal, S. and Basu, S.K. (1989) *Science* 244, 705–707.