Chapter 6

Discussion

Attempts have been made to target drugs exclusively to tumor cells using various carriers such as specific antibodies, liposomes, DNA, LDL, etc. with varying degrees of success (2-5). Incomplete specificity for cancer cells and inefficient internalization of the drug conjugate still remain major drawbacks of these approaches (4). In the present thesis, we have worked out an alternative modality for delivering drugs specifically to cancer cells exploiting receptor-mediated endocytosis of macromolecular drug conjugates.

6.1 Construction of the selective drug delivery system

The first major step in the formulation of a selective drug delivery system using a macromolecular carrier involves the conjugation of a cytotoxic agent to the carrier which can be recognised by specific determinants present exclusively on the cancer cells. For this purpose proteins, polysaccharides or synthetic polymers have been used (2). In devising a suitable carrier system for drug delivery following criteria need to be fulfilled, viz; i) the desired properties of the carrier or the activity of the drug should be unaffected, ii) the conjugate should contain optimal amount of the drug and the linkage between the drug and the carrier should be cleavable at the
target site, iii) it should not form large aggregates, iv) the method should be simple and reproducible, and v) reactive groups should be available on both the molecules. Accordingly, different types of reagents have been used for coupling of drugs with the carrier depending upon the functional group involved. In the present investigation MBSA has been used as a carrier which is recognised by specific receptors present on the target cells, viz., neoplastic cells of macrophage lineage. After high affinity binding, MBSA is internalized and subsequently degraded in the cellular lysosomes. Thus, this molecule fulfills two of essential criteria as a prospective carrier, viz., specificity for the target cells and biodegradability. Maleylation usually occurs in the lysyl residues of protein and is stable at neutral and alkaline pH (220). A number of carboxyl groups generated by the maleylation of the protein is useful for the conjugation of drugs having amino groups. Furthermore, maleylation of BSA can be controlled so that a number of amino group on the molecule can also be made available for the conjugation with the drug of choice.

6.2 Kinetics of uptake and degradation of drug conjugate by J774A.1 cells

In the present investigation, we have shown that MBSA-DNM is taken up by the tumor cells of macrophage lineage in a saturable fashion suggesting that the conjugate is recognised by a limited number of binding sites present on the cell surface (Fig. 6). Binding of $^{125}\text{I}$-MBSA-DNM was inhibited by MBSA but not by free DNM, indicating that the drug conjugate is recognised by MBSA binding sites (Fig. 7). This high affinity binding is followed by degradation of the drug conjugate (Fig. 8). The lysosomal inhibitor, monensin, inhibits the degradation of the drug conjugate reflecting that after initial binding with the cell surface receptors, MBSA-DNM is internalized and degraded in the lysosomes (Fig. 9). Moreover, when J774A.1 cells were allowed to bind $^{125}\text{I}$-MBSA-DNM at 4°C and then warmed to 37°C, acid soluble degradation products were detectable in the culture medium within 30 minutes and all the conjugate that entered the cell were degraded within 150 minutes (Fig. 10). This rapid
uptake process resembles the fate of acetylated LDL taken up through the receptors on macrophages indicating that the MBSA-DNM binding site is functionally adapted to mediate the uptake and degradation of molecules that bind to it. This rapid degradation affects only the molecule that is attached with the binding sites and is not due to a ligand mediated stimulation of non-specific fluid-phase endocytosis. This follows from the observation that unlabelled MBSA or MBSA-DNM did not accelerate the degradation of $^{125}\text{I}-\text{MBSA-DNM}$. The rapid uptake and degradation of $^{125}\text{I}-\text{MBSA-DNM}$ by J774A.1 cells were competed by MBSA, suggesting that the drug conjugate is taken up through polyanion binding sites present on tumor macrophages.

The results presented in Table 7 show that the degradation of radiolabelled drug conjugate is inhibited by MBSA, fucoidin, dextran sulphate, polyinosinic acid and polyguanylic acid at relatively low concentrations. In contrast, other negatively charged compounds, *viz.*, fetuin, chondroitin sulphate A, polyadenylic acid, polycytidylic acid and heparin which are known to be ineffective as competitors for the scavenger receptor system (167) require much higher concentrations to bring about inhibition of the degradation of $^{125}\text{I}-\text{MBSA-DNM}$ to the same extent. These data, taken together, indicate that the drug conjugate is taken up by the macrophage tumor cells through the scavenger receptors which recognise these ligands. The scavenger receptor pathway has been reported to consist of at least three distinct molecular entities with varying affinities for different ligands (178). The cross competition experiments reported here, however do not clearly indicate which of these receptors is involved in the high affinity uptake of MBSA-DNM drug conjugate.

In order to determine the cell type specificity of the drug conjugate, we measured the degradation of $^{125}\text{I}-\text{MBSA-DNM}$ by different transformed cell lines. The results presented in Fig. 12 show that only the tumor cells of macrophage lineage like J774A.1, P388D1 and IC-21 can degrade significant amounts of $^{125}\text{I}-\text{MBSA-DNM}$ at $37^\circ\text{C}$. In contrast, nonmacrophage tumor cells like EL-4 (T-lymphoma), L929 (fibroblast), Bowes melanoma (epithelial cells) and CHO were unable to take up and degrade MBSA-DNM suggesting that the cell lines are deficient in scavenger receptors.

The kinetic studies of uptake and degradation of $^{125}\text{I}-\text{MBSA-DNM}$ suggest that this drug conjugate is specifically recognised by the tumor cells of macrophage lineage
which express scavenger receptors on their surface. High affinity binding of the ligand to the cell surface receptors is followed by rapid internalization of the receptor-ligand complex. The internalized ligand is probably dissociated from the receptor in the endosomes followed by return of the receptor to the cell surface for multiple rounds of ligand delivery inside the cells while the ligand is degraded in the lysosomes.

6.3 Status of scavenger receptor on multidrug resistant tumor cells

Development of resistance to the administered drugs poses a major problem in cancer chemotherapy. Drug resistant phenotype is often associated with decreased intracellular drug accumulation because of increased efflux or decreased influx of the drug. In order to determine if the drug resistance in macrophage tumor cells can be circumvented by receptor mediated drug delivery, we have developed a DNM-resistant variant of J774A.1 cells by culturing the J774A.1 cells in presence of increasing concentrations of daunomycin. The resultant cells, named JD100, also showed increased resistance to doxorubicin, methotrexate, vincristine, 5-flurouracil and mitomycin C as shown in Table 9. Among these drugs, DNM, doxorubicin and vincristine are known to be transported out of the cells by the P-glycoprotein efflux pump (221,222). But other anticancer agents like methotrexate, 5-flurouracil and mitomycin C are known to be poor substrates for the P-glycoprotein. Membrane transport studies have demonstrated that calcium channel-blocking drugs such as verapamil bind directly to the P-glycoprotein, thus permitting increased intracellular drug accumulation (196). We found that in presence of verapamil (20 μg/ml), DNM, doxorubicin, and vincristine could exert cytotoxic effect on the JD-100 cells whereas methotrexate and 5-flurouracil did not show significant cytotoxic activity even in presence of verapamil. These results indicate that the multidrug resistance phenotype displayed by the JD100 cells is of a complex type and might involve P-glycoprotein-dependent and independent mechanisms.

To determine the status of scavenger receptors on the DNM-sensitive and resistant
cells, we compared the ability of J774A.1 and JD100 cells to bind and degrade $^{125}$I-MBSA-DNM at 4°C and 37°C, respectively. Data presented in Fig. 18-20 shows that $^{125}$I-MBSA-DNM is processed by both the cell types in a similar fashion reflecting that the scavenger receptors on both the cell types are similar in content as well as function. Therefore, it might be possible to utilize the scavenger receptor system for intracellular delivery of drugs to the multidrug resistant JD-100 cells.

### 6.4 Cytotoxic activity of MBSA-DNM: *in vitro* studies

The data in Fig. 13 show that MBSA-DNM brings about a rapid and total inhibition of the ability of J774A.1 cells to synthesize DNA as measured by $^{3}$H-thymidine incorporation. In contrast, free DNM under similar conditions cause a transient inhibition of DNA synthesis from which cells can recover and resume DNA synthesis at rates comparable to the untreated control. Conjugated DNM at 0.1 μM caused 50% killing of the receptor bearing J774A.1 cells whereas only 0.5% of these cells were eliminated by the same concentration of free DNM. Therefore, the drug conjugate was about 100-fold as effective as the free drug in killing the receptor bearing cells. Furthermore, receptor negative tumor cells remained unaffected by the same concentrations of the drug in conjugated form indicating the selectivity of the drug-conjugate in eliminating the tumor cells bearing the scavenger receptors (Fig. 15). The cytotoxic activity of drug conjugate results from its efficient uptake through scavenger receptor. This is indicated by the fact that in presence of polyguanylic acid, a known high affinity ligand for the scavenger receptor, the drug conjugate does not exert its cytotoxic effect (Table 8). MBSA-DNM treated cells were unable to form monolayers in culture (Fig. 14). But the cells treated with similar concentrations of DNM in free form could form monolayers. Results presented in Fig. 16 show that the *ex vivo* MBSA-DNM treatment of the cells derived from the J774A.1 intraperitoneal tumors abolishes their ability to form tumor in BALB/C mice, whereas such cells treated with free DNM under identical condition retain the ability to form tumor in
BALB/C mice as the untreated cells. The substantially enhanced cytotoxicity at low concentrations of MBSA-DNM compared to the free drug presumably results from the high efficiency of receptor-mediated uptake and lysosomal degradation of the drug conjugate permitting rapid build up of pharmacologically effective intracellular concentration of DNM (or a bioactive derivative). However, we have not excluded the possibility that the intracellular product released after receptor mediated uptake of MBSA-DNM could have higher cytotoxic activity than the free drug.

Exposure of J774A.1 cells to increasing concentrations of DNM led to the establishment of a multidrug resistant variant of these cells, named JD-100. Compared to the parent cells, the JD-100 cells were significantly more resistant to DNM, doxorubicin, vincristine, methotrexate and 5-flurouracil. Addition of verapamil to the culture medium reversed the resistance of JD-100 to DNM, doxorubicin and vincristine whereas the resistance to methotrexate and 5-flurouracil was unaltered. These results suggest that the multidrug resistance of JD-100 cells was due both to P-glycoprotein-dependent as well as independent mechanisms. The data in Fig 21 show that JD-100 cells were resistant to 0.3 μM free DNM whereas MBSA-DNM at 0.3 μM suppressed the ³H-thymidine incorporation by 50% as compared to the untreated controls. Therefore, scavenger receptor-mediated delivery of DNM could circumvent the resistance to DNM developed in these cells. Attempts have been made to overcome P-glycoprotein mediated resistance to anticancer drugs using combination therapy with verapamil (207,208). However, the narrow therapeutic window of verapamil action cause significant degrees of heartblock and hypertension which limit the utility of this combination therapy (209,210). Immunotoxins directed against the P-glycoprotein can also selectively kill multidrug resistant cells (211). But the major drawbacks of the immunotoxin approach arise from the facts that P-glycoprotein is also expressed in several normal tissues (212) and it is difficult to attain therapeutic concentrations of the drug in the solid tumors in vivo due to inefficient internalization of immunotoxins by the target cells. The receptor-mediated intracellular delivery of the drug to the multidrug resistant cells shown by our studies could provide distinct advantages over conventional chemotherapy.
6.5 In vivo antitumor efficacy of MBSA-DNM over free DNM

In vivo results described in the present investigation demonstrate that MBSA-DNM is rapidly cleared from the circulation and accumulates in the tissues rich in macrophages (Fig. 23, 24). These results indicate the possibility of selective delivery of drug to macrophages in vivo using the scavenger receptor system. Since the normal macrophages are terminally differentiated and do not divide, conjugates such as MBSA-DNM which affects only the proliferating cells, are not likely to affect the functional roles of the mature normal macrophages in vivo. Moreover, previous studies with a conjugate of MBSA with the potent anticancer drug, methotrexate, did not elicit any adverse reactions in hamsters (124). The data in the Fig 29 show that 30 µg drug in the conjugated form totally suppressed the J774A.1 tumor in BALB/C mice even when the treatment was started 7 days after tumor transplantation. At lower dosage, the antitumor activity of the drug conjugate was more pronounced (Fig. 25–27). At the dosage of drug required for achieving 100% tumor suppression in the conjugated form, the free drug did not show any antitumor activity and the mass of tumor remained as in the untreated control animals. It was significant that 30 days after tumor transplantation all the mice treated with the free drug developed tumor comparable to those in control animals and died within 40-45 days. In contrast, five mice in the experimental group (n=6) treated with the drug conjugate remained tumor free and 80% of them survived throughout the observed period (70 days). MBSA did not show any antitumor activity. Similar results was also obtained in case of intraperitoneal tumors treated with the drug conjugate. Table 9 shows that mice bearing intraperitoneal tumor treated with MBSA-DNM survived throughout the experimental observation of 230 days, in contrast to 40 days in case of tumor treated with the same concentration of free DNM. Although, antibodies reacting with MBSA-DNM was elicited during the therapy, the antitumor activity of the conjugate in suppressing tumor growth was not affected (Table 11). Use of maleylated carrier derived from homologous albumin should reduce this immune response. We have shown that single administration of the drug in the conjugated form can also
suppress the growth of the tumor in mice (Fig. 28). This may be of further help in reducing the immune response to drug conjugate. It is pertinent to mention that all the animals which received MBSA-DNM were healthy throughout the experimental period indicating no apparent toxic effect of the drug conjugate.

Concurrent with our work, a few attempts have been made for selective drug delivery to cancer cells using various receptor systems for lipoproteins, growth factors etc. (133–141). However, since these receptors are also found on most normal cells, it is likely that the drug delivered through these receptors would not be exclusively limited to the tumor cells. Nevertheless, low-density lipoprotein receptor mediated drug delivery has received most attention so far, but the major limitation of this carrier is that only lipophilic drugs can be incorporated into the LDL molecule (139). Moreover, lipoproteins are complex molecules with limited stability and are difficult to formulate into stable pharmacological preparations. In contrast, MBSA as a drug carrier is attractive because of simplicity of preparation, longer shelf life, ease of sterilization and formulation into apyrogenic preparation. Our study of receptor-mediated delivery of an anticancer agent to macrophage tumor cells demonstrate the superior antitumor efficacy of the drug conjugate on suppression of solid tumors in an animal model without any apparent toxic effect of the drug conjugate. This modality merits serious consideration in the search for new chemotherapeutic agents for combating histiocytic malignancies which are usually fatal and difficult to control by conventional chemotherapeutic measures.