Chapter 4

Materials and Methods

4.1 Materials

4.1.1 Chemicals

All the drugs used in this study like daunomycin, doxorubicin, methotrexate, 5-fluouracil, mitomycin C, vincristine etc. were purchased from Sigma Chemical Co., USA. Other fine chemicals like, bovine serum albumin (BSA), maleic anhydride, chloroquine, monensin, polyinosinic acid, polyguanylic acid, polycytidilic acid, fucoidin, were also purchased from Sigma Chemical Co. USA. Dextran sulphate was obtained from Serva. Sephadex G-50 was purchased from Pharmacia Fine Chemicals, Sweden. Bicinchoninic acid (BCA) protein assay reagent was from Pierce Co. For the preparation of scintillation fluid, 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazolyl)-benzene (POPOP) were purchased from Beckman.

For the ELISA assay, horse radish peroxidase (HRP) conjugated rabbit antimouse IgG was obtained from Sera-Lab. Trypsin and skim milk were from DIFCO. Orthophenylenediamine (OPD) was purchased from Dakopatts, whereas Tween-20 from Sigma. Other reagents and chemicals used during this study were of analytical grade.
4.1.2 Cell lines

Mouse tumor cell lines of monocyte-macrophage origin, J774A.1, P388D1 and IC-21 were obtained from American Type Culture Collection, USA. Bowes melanoma, a human cancer cell line, was kindly provided by Desire Collen of University of Leuven, Belgium. EL-4 (mouse T cell lymphoma) and L929 (mouse fibroblast) cell lines were obtained from Peter H. Krammer of German Cancer Research Centre and Indira Nath of All India Institute of Medical Sciences, New Delhi, respectively.

The tissue culture media used like Roswell Park Memorial Institute (RPMI) 1640, Eagle’s Minimum Essential Medium (MEM), Dulbecco’s Modified Eagle Medium (DMEM), HAMS and Hanks Balanced Salt Solution (HBSS) were purchased from Gibco Laboratories, Grand Island, N.Y. Fetal Calf serum (FCS) was obtained from Sera-Lab., U.K. Penicillin G, streptomycin sulphate, Na-bicarbonate, 2-mercaptoethanol were from Sigma Chemical Co., USA. Glutamine and Na-pyruvate were purchased from Serva. All tissue culture disposables and sterile plasticware were purchased from Costar and Nunc, U.S.A.

The radioactive isotopes used in this study viz., carrier free Na ($^{125}$I) and $^{3}$H-thymidine were purchased from Bhabha Atomic Research Centre, Bombay, India, at regular intervals.

4.1.3 Animals

The animals used during this study were 4-6 weeks old female BALB/C mice obtained from National Institute of Nutrition, Hyderabad at regular intervals and were maintained in the animal house of Institute of Microbial Technology, Chandigarh.

4.2 Methods

4.2.1 Culture of different cell lines

J774A.1, the transformed mouse macrophage cell line mostly used during the study, was cultured and maintained in sterile DMEM containing 10% heat inactivated fetal
calf serum, 3.7 g/l of Na-bicarbonate, 2 mM glutamine, 100 mg/l streptomycin and 70 mg/l of penicillin at 37°C in 5% CO₂-95% air atmosphere. The cells were grown as monolayers on plastic tissue culture flasks with a flat surface. The cells grew exponentially with a doubling time of 16-17 hours and were fed twice a week to maintain logarithmic growth. Viability of the cell line was periodically checked by Trypan blue exclusion. Whenever needed, desired number of cells were dispensed into 24-well tissue culture plastic plates and incubated as required.

The other cell lines used were cultured and maintained as described above. But the media used were different for different cell lines, viz. DMEM for L929, RPMI-1640 for P388D1, IC21 and EL-4, MEM for Bowes melanoma and HAM for CHO with 10% FCS, 2 mM glutamine and 50 µg/ml gentamycin at 37°C in 5% CO₂ - 95% air atmosphere.

4.2.2 Freezing of cells
Cells were harvested and pelleted down by centrifugation at 1000 rpm for 10 minutes using Sorvall RC3C Centrifuge. The cell pellet was resuspended either in DMEM, RPMI-1640 or MEM (depending on the cell) containing 10% DMSO and 30% FCS at a concentration of 1 x 10⁷ cells per ml of medium. These cells were aliquoted into freezing vials which were kept immediately at -20°C (Kelvinator, India) for two hours which was followed by -70°C (Revco) overnight. On the next day, cells were stored in liquid nitrogen (Cryomed). Whenever required, cells were taken out, thawed at 37°C, washed and resuspended in respective medium containing 10% FCS and was grown in the same medium at 37°C, 5% CO₂ - 95% air atmosphere.

4.2.3 Maleylation of Bovine Serum Albumin
Maleylation of Bovine Serum Albumin (BSA) was done by addition of maleic anhydride to the protein solution at constantly maintained alkaline pH (8.0) (213). Briefly, 1 gm of BSA was dissolved in 40 ml of glass distilled water and the pH was maintained at 8.0 by the addition of NaOH in the constantly stirring solution. Maleic anhydride (2.5 gm) was added over one hour to the continuously stirred reaction mixture at
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room temperature. The pH of the reaction mixture was maintained constantly at 8 by the addition of 1M NaOH as required until there was no further liberation of acid. The reaction mixture was dialysed against 15 litres of 20 mM cold phosphate buffered saline (PBS), pH 7.2 and stored in aliquotes at -20°C until used.

4.2.4 Estimation of protein

Biuret method (214) was used to estimate the protein content of maleylated bovine serum albumin (MBSA). In practice, 0.1 ml of a solution containing 0.1 to 1 mg of protein was added with 0.4 ml of Biuret reagent, swirled to mix and kept at room temperature (20–25°C) for 30 minutes. The absorbance, was measured at 560 nm against the blank having 0.4 ml biuret reagent and 0.1 ml of water or of an appropriate salt solution. A calibration curve was prepared with a solution of BSA and concentration of protein in the sample was estimated by comparison with the standard curve.

4.2.5 Electrophoretic mobilities of proteins

Agarose gel electrophoresis was done to measure the electrophoretic mobilities of MBSA and BSA (215). For this purpose, a 6 cm x 10 cm x 0.4 cm of 0.7% gel was cast in 0.05 M Na-barbital buffer, pH-8.4. Then 100 µg of protein solution in 20 µl of loading buffer (0.01% Bromophenol blue in 10% glycerol) was loaded in respective well and electrophoresis was carried out at 30 mA (Pharmacia Power supply) for 4 hours in the same buffer using Biorad gel apparatus. The gel was stained in 0.1% Coomassie blue in 45% methanol and 5% acetic acid and subsequently destained with 45% methanol and 5% acetic acid. Electrophoretic mobilities of the proteins were calculated from the Rf values.

4.2.6 Preparation of MBSA-DNM drug conjugate

MBSA was coupled to daunomycin (DNM) using glutaraldehyde as described (85). Briefly 3 mg of MBSA was thoroughly mixed with 400 µg of DNM in 1 ml PBS. One hundred µl of 0.1% glutaraldehyde was added dropwise to the reaction mixture to
achieve final concentration of 0.01% glutaraldehyde and kept at stirring condition for 15 minutes at room temperature. The reaction was stopped by adding 50 μl of 1 M lysine.

The conjugate was recovered from the residual excess reactants by Sephadex G-50 column chromatography. For this purpose, swollen Sephadex G-50 equilibrated with 20 mM phosphate buffered saline (0.15 M), pH 7.2, was packed in a 1.5 cm x 120 cm. column (bed volume, 110 ml) and washed with PBS. The reaction mixture (1 ml) was loaded on the column and eluted with the same buffer at a flow rate of 10 ml/hour at room temperature and 1 ml fractions were collected. DNM content of the conjugate was determined by measuring the absorbance at 495 nm (85) using spectrophotometer. The void volume fractions were pooled and dialysed extensively against PBS.

4.2.7 Radioiodination of MBSA-DNM

The procedure for preparing radioiodinated MBSA-DNM was based on Bilheimer’s modification of iodine monochloride catalysed method of Mac Farlane (6). For the preparation of iodine monochloride, 150 mg of sodium iodide dissolved in 8 ml of 6 N HCl, were mixed with 99 mg of sodium iodate (anhydrous) dissolved in 2 ml of water. The iodate solution was forcibly injected into the iodide - HCl solution in order to avoid precipitation of iodine. Water was added to dilute the mixture to a final volume of 40 ml and was shaken in a glass-stoppered cylinder with 5 ml of carbon tetrachloride. The upper organic phase was discarded and residual carbon tetrachloride removed by aerating the aqueous phase with moist air for 1 hour. Water was added to increase the volume of aqueous phase to 45 ml. This solution was 33 mM with respect to iodine monochloride and approximately 1 N with respect to HCl. This stock solution was diluted with 12.5 volumes of 2 M NaCl immediately before iodination.

The iodination reaction was performed at 4°C behind the lead bricks in a fume hood. For iodination, 250 μl of 1 M glycine-NaOH buffer and 250 μl of protein (2-5 mg) in phosphate buffer were taken in a glass tube. To this protein solution, 2 mCi of Na $^{125}$I was added along with 125 μl (2.64 mM) diluted iodine monochloride solution.
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The tube was vortexed for 2 seconds during the addition of iodine monochloride which was followed by incubation of the reaction mixture for 5 minutes on ice. Final volume of the reaction mixture (1 ml) was adjusted with the addition of 375 μl of PBS. The labelled conjugate was separated from low molecular weight reactants using PD-10 (Sephadex G-25 column of Pharmacia) column followed by extensive dialysis against cold PBS for 24 hours. More than 99% of the radioactivity was precipitated by 10% trichloroacetic acid and the specific activity of the radiolabeled drug conjugate was 120 cpm/μg protein.

4.2.8 Estimation of cellular protein

Cellular protein contents were measured using method of Smith et al. (216) using BSA as standard. BCA reagent A (1 ml) was mixed with 50 ml of reagent B, 2 ml of the mixture was added to 0.1 ml of cell extract (0.1 ml). After incubation at 37°C for 30 min, the absorbance was measured at 562 nm.

4.2.9 Assay of binding of $^{125}$I-MBSA-DNM by J774A.1 cells at 4°C

For binding experiment, each well of 24 well tissue culture plates received 1 ml of medium A (DMEM supplemented with 10% FCS, 2 mM glutamine and 50 μg/ml gentamycin as described earlier) containing $5 \times 10^5$ cells of J774A.1 and were incubated at 37°C in a humidified incubator with 5% CO$_2$ - 95% air atmosphere. After 24 hours, the monolayers were washed twice with FCS free medium A and replaced by 1 ml of ice-cold medium B (medium A without bicarbonate and FCS but containing 1 mg/ml of bovine serum albumin) containing different concentrations of $^{125}$I-MBSA-DNM and the monolayers were incubated at 4°C for 2 hours to reach steady state.

After 2 hours, the monolayers were washed rapidly two times with 2 ml of ice cold PBS containing 1 mg/ml of BSA and subsequently twice with 1 ml of ice cold PBS. The cells were dissolved in 1 ml of 0.1 (N) NaOH. Appropriate aliquotes of the cell extract were used for determination of the cell associated radioactivity in a gamma-counter (Beckman, model Gamma 5500, U.S.A.). Cellular protein was estimated
using an aliquote of cell suspension by BCA method (216).

4.2.10 Assay of uptake and degradation of $^{125}$I-MBSA-DNM at 37°C.

J774A.1 cell monolayers prepared in 24 well tissue culture plates as described earlier, were washed twice with FCS free medium A. Each well then received 1 ml of pre-warmed medium C (medium A without FCS but containing 1 mg/ml bovine serum albumin) containing indicated concentrations of $^{125}$I-MBSA-DNM and the monolayers were incubated at 37°C for 5 hours or various other periods. The cells were transferred to 4°C cold room, the medium was immediately removed and monolayers were washed as above. Cells were dissolved in 1 ml of 0.1 N NaOH and aliquotes were used for determining cell associated radioactivity and the content of cellular protein as above. Results were expressed as of MBSA-DNM per mg of cellular protein. The medium was saved for measurement of degradation of $^{125}$I-MBSA-DNM.

Degradation of $^{125}$I-MBSA-DNM was assayed as described in (217). For measuring the degradation of $^{125}$I-MBSA-DNM, cells were incubated with indicated concentrations of $^{125}$I-MBSA-DNM for respective times as described in preceding section. Then 0.5 ml of the medium was removed from each cell monolayer, and was added to a microfuge tube containing 125 μl of 50% TCA to precipitate undegraded $^{125}$I-MBSA-DNM. After incubation at 4°C for at least 30 minutes, the acid precipitable material was removed by centrifugation using BiofugeA (Heraeus Sepatech). An aliquote (0.4 ml) of the TCA soluble supernatant was mixed with 7 μl of 40% potassium iodide as a carrier, 15 μl of 30% hydrogen peroxide and kept at room temperature for 5-10 minutes. Then 1 ml chloroform was added to each tube to extract any free iodine into chloroform layer by vortex agitation. After 15 minutes at room temperature, an aliquote of 250 μl was removed from the upper aqueous layer and its content of radioactivity was determined. This aqueous material consists of ($^{125}$I) monoiotyrosine released from the lysosomal degradation of $^{125}$I-MBSA-DNM. Similar concentrations of $^{125}$I-MBSA-DNM was incubated and processed in similar ways but without any cells, used as control. The TCA soluble radioactivity obtained from the no cell
blank was subtracted from that obtained in parallel incubations with cell monolayers. All the values were obtained from the average of triplicate determinations and represented as ng of MBSA-DNM degraded per mg of cell protein. In competition experiments, competing compounds like MBSA, DNM, fucoidin, dextran sulphate, polyinosinic acid, polyguanylic acid were incubated at 37°C in the medium along with 125I-MBSA-DNM.

4.2.11 Internalization of bound 125I-MBSA-DNM by J774A.1 cells

Monolayers of J774A.1 cells in 24 well tissue culture plates were prepared as above. Each monolayer containing 1 ml ice cold medium B and 6 μg/ml of 125I-MBSA-DNM was incubated at 4°C. After 2 hours, cells were washed twice with ice cold PBS to remove the unincorporated radioactivity and the medium was replaced by 1 ml prewarmed (37°C) medium C (medium A without FCS but containing 1 mg/ml BSA) and the monolayers were incubated at 37°C in the CO2 incubator. At indicated time points, the amount of radioactivity associated with the cells and the amount of TCA-soluble radioactivity in the medium were determined in triplicate dishes, as described above. Cellular protein content was estimated by using BCA reagent and the results were expressed as ng of MBSA-DNM degraded per mg of cell protein.

4.2.12 Development of drug resistant J774A.1 cells

For the development of DNM resistant J774A.1 cells, the same cells were cultured in the presence of a sublethal concentration of DNM in the cell culture medium. JD-100 cells, resistant to 100 ng of DNM were obtained by continuous exposure to stepwise increasing drug concentrations from 25 ng to 100 ng. JD-100 cells were maintained in DMEM containing 100 ng per ml DNM, 20% FCS for the whole experimental period, at 37°C in humidified CO2 incubator. The DNM resistant phenotype expressed by these cells was stable during the course of this study. JD-100 cells became dependent on DNM for normal growth and removal of DNM resulted in some cell death and unusual morphology until the cell population stabilized after 7-10 days.
4.2.13  In vitro cytotoxic activity of MBSA-DNM and free DNM

The cytotoxic activity of the drug conjugate was assayed by three methods viz. (i) $^{3}$H-thymidine uptake assay (218), (ii) ability of the drug-treated cells to form monolayers and (iii) ability of the drug-treated cells to form tumors in mice.

i) $^{3}$H-thymidine uptake: Briefly, $1 \times 10^5$ cells per well were seeded in 24-well tissue culture plates and incubated overnight in 1 ml of medium A at 37°C in the presence of 5% CO$_2$ 95% air atmosphere. The cells were washed and exposed to indicated concentrations of DNM either in free or conjugated form in 1 ml medium A for 30 minutes at 37°C. The cells were then washed thrice with drug-free medium A and incubated at 37°C in drug-free medium A for the indicated intervals. At respective time points, each well received 5 μCi/ml of $^{3}$H-thymidine and the incubation continued at 37°C. After 3 hours, the cells were washed 4 times with medium A to remove the unincorporated radioactivity. The cells were solubilized in 0.5 ml of 0.1 N NaOH and an aliquote was used to determine $^{3}$H-thymidine associated with the cells using an LKB 1211 Rackbeta counter.

(ii) Ability to form monolayer: J774A.1 cells ($2.5 \times 10^4$ cells per well) were seeded on 24 well tissue culture plates on day 0 and incubated at 37°C in a humidified incubator with 5% CO$_2$ - 95% air atmosphere in 1 ml medium A. On day 1, the monolayers were washed and incubated in 1 ml medium A containing different concentrations of DNM either in free or conjugated form for 30 minutes at 37°C. The cells were washed three times with medium A and incubated in 1 ml of medium A at 37°C in a humidified incubator. On day 4, the monolayers were washed with PBS, fixed at room temperature with 3% formaldehyde and stained with 0.1% crystal violet for 5 minutes.

(iii) Ability of drug treated cells to form tumor in mice: Cells isolated by lavage of 10 ml PBS per mouse, from the peritoneal cavity of donor BALB/C mice bearing J774A.1 intraperitoneal tumors, were washed in PBS and suspended in medium A at a concentration of $1 \times 10^6$ cells/ml, and incubated in presence of 0.4 μM DNM either in free or conjugated form for 5 hours at 37°C in a humidified incubator with 5%
The mice developed ascites within 10 days of transplantation and all the animals died within 30 days. Injection of the ascitic cells subcutaneously (5 x 10^6 cells/site) in 6-8 weeks old inbred BALB/C mice, led to formation of solid subcutaneous tumors and death of the animals within 25-30 days if untreated.

**4.2.17 Treatment of intraperitoneal and subcutaneous tumor with MBSA-DNM or DNM.**

In order to test the effect of administering MBSA-DNM or DNM on the formation of the intraperitoneal tumors, 5 x 10^5 J774A.1 cells were transplanted intraperitoneally to each animal on day 0. Indicated dosages of DNM either in free or conjugated forms were injected intraperitoneally to these animals for 4 consecutive days, on days 1, 2, 3 and 4. The mean survival time of 6-8 mice in each group were determined. In case of subcutaneous solid tumors 5 x 10^6 J774A.1 cells were transplanted into the subcutaneous tissues of each BALB/C mice on day 0. Indicated dosages of both free as well as conjugated drug was injected intraperitoneally into the mice on alternate days, on days 1, 3, 5 and 7. Control animals received only the same volume of PBS. The size of the tumors were determined on day 30.

In another set of experiments, the effect of DNM (free or conjugated) on the growth of tumor was determined. The mice transplanted with J774A.1 tumor cells on day 0 were treated with 30 \mu g of DNM either in free or conjugated forms on days 1, 3, 5, 7. The growth of tumor was observed at 4 day intervals upto 35 days.

**4.2.18 Treatment of preformed subcutaneous tumor with free and conjugated daunomycin**

In order to determine the effect of administered daunomycin on preformed tumors, 5 x 10^6 J774A.1 cells per animal were injected on day 0. After 7 days, when the tumor weight was about 380 mg, treatment was started with 4 injections (day 7,9,11,13) of 30 \mu g of DNM in free or conjugated form on alternate days. Tumor size was measured starting from the day of tumor transplantation.
plate was covered with a plate sealer and kept at 37°C for 1 hour or at 4°C overnight. Then the plate was washed well with PBS containing 0.05% Tween 20. Unoccupied sites on the plates were blocked with 3% skim milk in PBS at 4°C overnight or 37°C for 1 hour. The wells were then washed and incubated with the respective serum in different dilutions, mainly 1:100, 1:500 and 1:1000 in 1% skim milk in PBS for 2 hours at 37°C. Unreacted antibody was washed off and subsequently the wells were treated with horse radish peroxidase conjugated rabbit antimouse IgG at 1:1000 dilution in PBS containing 3% skim milk for 180 minute. The wells were again washed with PBS containing 0.05% Tween 20 and finally colour was developed with 0.2 mg/ml orthophenylenediamine (OPD) in 20 mM PBS, pH 7.2 containing hydrogen peroxide. The plate was protected from light. The reaction was stopped by adding 100 μl of 7% H₂SO₄ to each well and its absorbance at 492 nm was measured using an ELISA reader.