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ENHANCEMENT OF TUMOURICIDAL ACTIVITY OF DAUNOMYCIN BY RECEPTOR-MEDIATED DELIVERY

IN VIVO STUDIES

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Abstract—A conjugate of the antineoplastic drug daunomycin (DNM) with maleylated bovine serum albumin (MBSA) bound to the transformed cells of a macrophage lineage with high affinity and saturation kinetics through scavenger receptors present on the surface of macrophages. Binding of MBSA–DNM by these cells led to efficient internalization and degradation of the ligand. When injected into mice, the drug conjugate was cleared rapidly from the circulation and accumulated in the macrophage-rich tissues, viz. liver, lung and spleen. MBSA–DNM suppressed the growth of J774A.1 tumour cells in BALB/C mice at much lower dosages of DNM relative to the free form of the drug. Thus, 50% reduction of the tumour mass was elicited by 0.8 μg of DNM in the conjugated form, whereas approximately 28 μg of the drug in the free form was necessary to achieve a similar antitumour effect. These findings merit serious consideration in the development of new chemotherapeutic agents for the treatment of histiocytic malignancies that involve cells of macrophage lineage bearing the scavenger receptors.

Antibiotics of the anthracycline group [e.g. daunomycin (DNM)§ and doxorubicin] are highly effective in the treatment of human neoplastic diseases such as leukemias and sarcomas [1]. However, severe cumulative myocardial toxicity of these drugs limits their wider use in cancer chemotherapy for prolonged periods [2]. These adverse side-reactions possibly arise due to the fact that at therapeutically effective concentrations in the blood, the nontarget cells in the body are also exposed to the severe cytotoxic effects of these drugs. It is possible that such side-effects could be minimized if a modality of delivering drugs exclusively to cancer cells could be worked out, which would: (i) minimize the uptake of the drugs by nontarget cells, (ii) selectively deliver the drug only to the target cells at a relatively low concentration in the blood, and (iii) ensure efficient intracellular availability of the drug. Attempts have been made, with varying degrees of success, to deliver anthracycline antibiotics exclusively to the tumour cells using various carriers such as specific antibodies, liposomes, DNA, and low-density lipoprotein (LDL) [3–7]. Incomplete specificity for cancer cells and/or inefficient internalization of such drug conjugates still remain major drawbacks of these approaches [8].

We have sought to exploit the exquisite cell-type specificity and high efficiency of the process of endocytosis of macromolecules mediated by the "scavenger receptor" system [9], present predominantly on the cells of macrophage lineage, for developing an efficient modality for delivering drugs to these cells. This receptor system recognizes a variety of polyanionic macromolecules including maleylated bovine serum albumin (MBSA) [10]. After binding to these receptors, MBSA is internalized and degraded in cellular lysosomes to free amino acids with high efficiency. We have shown previously that conjugates of MBSA with the appropriate drugs show superior efficacy in eliminating the intracellular pathogens causing leishmaniasis and tuberculosis in comparison with the drugs in the free form [11–13]. Recently, we have also shown that MBSA–DNM preferentially eliminates transformed cells of macrophage lineage *in vitro* [14].

In the present investigation, we have demonstrated that MBSA–DNM is much more effective than free DNM in eliminating murine tumours of macrophage lineage *in vivo*. This approach is likely to be useful in the chemotherapy of histiocytic malignancies such as monocytic leukemia in which cells of macrophage lineage harbouring the scavenger receptors turn malignant [15].

EXPERIMENTAL METHODS

Materials. DNM, bovine serum albumin (BSA) and maleic anhydride were purchased from the Sigma Chemical Co. (St. Louis, MO). Tissue culture supplies were from the Grand Island Biological Co. (Grand Island, NY). Other reagents used were of analytical grade. The macrophage tumour cell line

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§ Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; DNM, daunomycin; FBS, fetal bovine serum; MBSA, maleylated BSA; PBS, phosphate-buffered saline; and TCA, trichloroacetic acid.

J774A.1 was purchased from the American Type Culture Collection. These cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) with 10% fetal bovine serum (FBS) and kept in a 5% CO_2–95% air atmosphere. J774A.1 tumours were developed and maintained in 6- to 8-week-old female BALB/C mice.

Preparation of MBSA–DNM conjugate. MBSA–DNM was prepared as described before [14] using glutaraldehyde [16]. The DNM content of the conjugate was determined by measuring the absorbance at 495 nm, and the protein was measured using bicinchoninic acid (BCA) reagent [17]. Stoichiometric estimation revealed that 3–4 mol of DNM was coupled per mol of MBSA.

Radioiodination of MBSA–DNM. MBSA–DNM was radioiodinated by iodine monochloride catalysed reaction [18]. More than 99% of the radioactivity was trichloroacetic acid (TCA)-precipitable, and the specific activity was 120 cpm/ng protein.

Binding of [^{125}I]MBSA–DNM to J774A.1 cells at 4°. Each well of the 24-well tissue culture plates received 1 mL of medium A (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine and 50 μg/mL gentamycin) containing 5×10^5 J774A.1 cells. The plates were incubated at 37° in a humidified incubator with a 5% CO_2–95% air atmosphere. After 24 hr, the monolayers were washed twice with FBS-free medium A. For binding experiments, 1 mL of ice-cold medium B (medium A without bicarbonate and FBS but with 1 mg/mL of BSA) containing different concentrations of [^{125}I]-MBSA–DNM was added to the wells and the monolayers were incubated at 4°. After 2 hr, the monolayers were washed three times with ice-cold phosphate-buffered saline (PBS) containing 1 mg/mL BSA and once with PBS. The cells were suspended in 1 mL of 0.1 N NaOH, and the amounts of radioactivity associated with the cells were determined in triplicate dishes as described before [18]. Cellular protein was estimated using an aliquot of the cell suspension by the BCA method [17].

For competition experiments, each monolayer received [^{125}I]MBSA–DNM (6 μg/mL) in the presence of the indicated concentrations of either free DNM or MBSA and was incubated at 4° for 2 hr. The cell-associated radioactivity was determined as indicated above.

Internalization and degradation of bound [^{125}I]-MBSA–DNM by J774A.1 cells. Monolayers of J774A.1 cells in 24-well tissue culture plates were prepared as above. Each monolayer received 1 mL of ice-cold medium B with 6 μg/mL of [^{125}I]MBSA–DNM and was incubated at 4°. After 2 hr, the cells were washed twice with ice-cold PBS to remove the unincorporated radioactivity and the medium was replaced by 1 mL of prewarmed (37°) medium C (medium A without FBS but containing 1 mg/mL BSA), and the monolayers were incubated at 37° in the CO_2 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. Cellular protein content was estimated by using BCA reagent and the results were expressed as nanograms of MBSA–DNM bound or degraded per milligram of cell protein. Each monolayer contained 90–100 μg protein/well.

Blood clearance and tissue distribution. Female BALB/C mice were infused intravenously with 1×10^7 cpm of [^{125}I]MBSA–DNM through the tail vein. The blood was collected in heparinized tubes at different time points by orbital puncture, and an aliquot was counted to determine the radioactivity present in the blood. Radioactivity present in the blood within 1 min after intravenous injection of [^{125}I]MBSA–DNM was taken as 100%.

To determine the distribution of [^{125}I]MBSA–DNM in various tissues, the mice were killed 10 or 20 min after intravenous administration of [^{125}I]-MBSA–DNM (1×10^7 cpm) and different tissues were dissected out. Tissues were washed in ice-cold Hanks' balanced salt solution (HBSS), and the content of radioactivity per gram of tissue was determined. Results were expressed as percentage of incorporation in the liver, arbitrarily chosen as 100%.

Transplantation of J774A.1 tumour in BALB/C mice. J774A.1 macrophage tumour cells were isolated from the peritoneal cavity of tumour-bearing BALB/C mice by injecting 10 mL of PBS. The cells were slowly aspirated out in 8–10 mL of lavage fluid and collected by centrifugation at 400 *g* for 10 min at 4°. The cells were washed, resuspended in PBS at a concentration of 5×10^7 cells/mL, and were injected subcutaneously (5×10^6 cells/site) in 6- to 8-week-old inbred BALB/C mice. The mice developed solid tumours within 3 days of transplantation, and all the animals died within 40–45 days.

Evaluation of tumour weight. At indicated time intervals during the course of tumour growth, the major and minor axes of the tumour were measured perpendicularly using a slide caliper by the same observer. Tumour weight was calculated by the formula [19].

$$\text{Tumour weight (mg)} = \frac{[\text{major (mm)} \cdot \text{minor (mm)}]^2}{2}$$

Treatment of tumour with MBSA–DNM or DNM. After transplantation of J774A.1 cells into the subcutaneous tissues of BALB/C mice, the indicated concentration of DNM in either free or conjugated form was injected intraperitoneally into the mice on indicated days. Control animals received only the same volume of PBS.

RESULTS

Binding of MBSA–DNM by J774A.1 cells. To measure the surface binding of MBSA–DNM, J774A.1 cells were incubated in the presence of different concentrations of [^{125}I]MBSA–DNM at 4°. MBSA–DNM bound to the cells with saturation kinetics (Fig. 1a). The half-maximal binding to the cells was achieved at approximately 2 μg/mL of [^{125}I]MBSA–DNM in the medium.

Binding of radiolabeled MBSA–DNM was inhibited effectively by MBSA but free DNM did not show any significant effect on the binding of the drug conjugate by J774A.1 tumour cells (Fig.

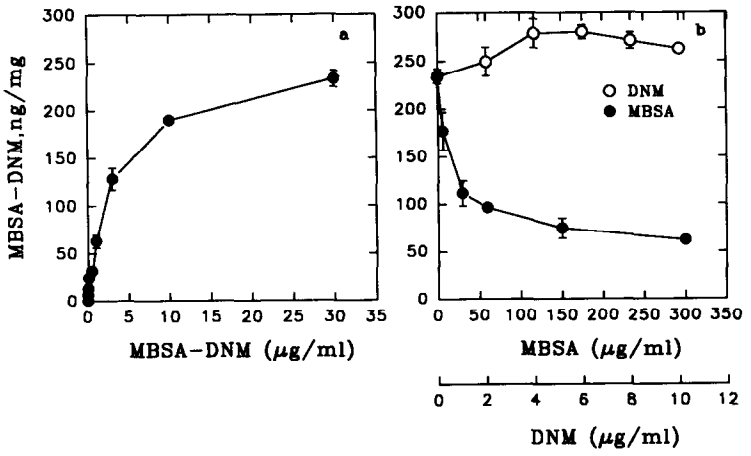

Fig. 1. (a) Binding of different concentrations of [^{125}I]MBSA–DNM by J774A.1 cells at 4° for 2 hr. Results are the means ± SD of three independent determinations. (b) Effects of MBSA and DNM on [^{125}I]MBSA–DNM binding by J774A.1 cells at 4°. Results are the means ± SD of three independent experiments.

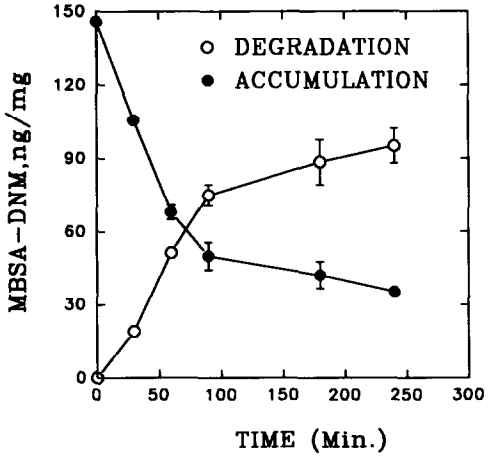

Fig. 2. Degradation of [^{125}I]MBSA–DNM at 37° previously bound by J774A.1 cells at 4°. Results are the means ± SD of three independent determinations and are expressed as ng of MBSA–DNM degraded or accumulated per mg of cell protein.

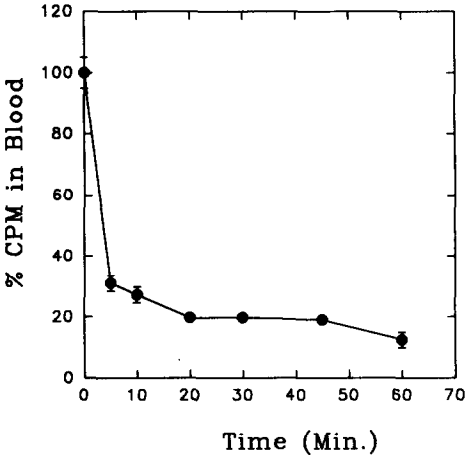

Fig. 3. Clearance of intravenously infused [^{125}I]MBSA–DNM from the blood of BALB/C mice. Results are the means ± SD of three independent experiments.

1b). Unlabeled MBSA–DNM was as effective a competitor as MBSA (data not shown).

Internalization of bound [^{125}I]*MBSA–DNM by J774A.1 cells.* To determine the kinetics of uptake of the drug conjugate by macrophage tumour cells, cells that had bound [^{125}I]MBSA–DNM at 4° were subsequently warmed to 37°. The cell-associated radioactivity declined rapidly and reached a steady-state in about 100 min. About 90% of the decline was due to the release of acid-soluble radioactivity in the medium from the cells after proteolytic degradation of the drug conjugate, a process that reached steady-state also by 100 min (Fig. 2).

Fate of the drug conjugate in BALB/C mice. When radioiodinated MBSA–DNM was injected intravenously into the BALB/C mice, about 70% of the radioactivity was cleared from the circulation within 5 min (Fig. 3). The data in Fig. 4 show that following rapid clearance from the blood compartment, the radioactivity was mainly associated with tissues rich in macrophage, viz. spleen, liver and lung.

In vivo *antitumour efficacy of MBSA–DNM.* J774A.1 tumour cells were transplanted into BALB/C mice on day 0. The indicated concentrations of DNM in either the conjugated or the free form were injected intraperitoneally on days 1, 3, 5 and 7. The weights of the tumours were determined on day 30.

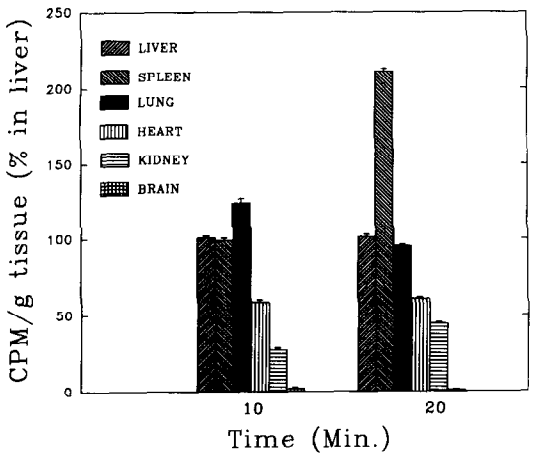

Fig. 4. Tissue distribution of intravenously injected [^{125}I]MBSA–DNM in BALB/C mice. Results are the means ± SD of three independent determinations. Contents of radioactivity in liver 10 and 20 min after injection of [^{125}I]MBSA–DNM were 168,500 and 187,000 cpm/g respectively.

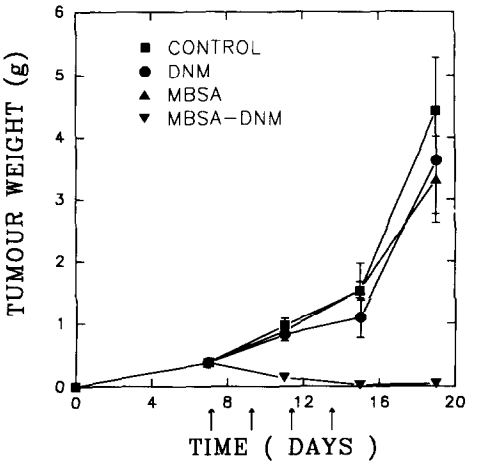

Fig. 6. Effects of MBSA–DNM and DNM on the growth of preformed J774A.1 tumours in BALB/C mice. J774A.1 cells were transplanted subcutaneously in BALB/C mice and allowed to form tumours. MBSA (3 mg protein), DNM (30 μg), or MBSA–DNM (30 μg DNM) was injected intraperitoneally in BALB/C mice on days 7, 9, 11, and 13. The results are expressed as mean weight (N = 6) of the tumours ± SEM.

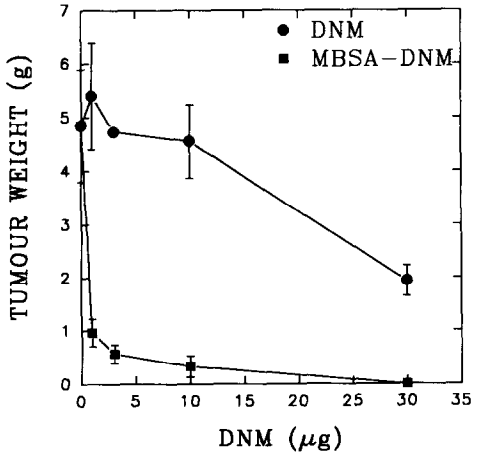

Fig. 5. Suppression of the tumour growth in BALB/C mice by different concentrations of DNM and MBSA–DNM. J774A.1 cells (5×10^6) were transplanted subcutaneously in BALB/C mice on day 0. Subsequently, animals were treated with different doses of DNM or MBSA–DNM intraperitoneally on days 1, 3, 5 and 7. The weights of the tumours were determined on day 30. Results are expressed as the mean weight of tumours ± SEM of 6–8 animals per group. The weight of the tumours in the control animals was 4.8 ± 1.8 g.

The data in Fig. 5 show that 30 μg of DNM in the conjugated form was needed for nearly total suppression of the tumour. In contrast, the same dosage of the free drug caused only 60% reduction in the weight of the tumour.

To determine the effect of administering DNM on preformed tumours, the J774A.1 cells were transplanted into BALB/C mice on day 0 and allowed to form a tumour in the animals. When the tumour weight reached about 380 mg, the tumour-bearing mice were administered PBS, DNM, MBSA or MBSA–DNM. As shown in Fig. 6, the size of the tumour increased rapidly in the PBS-treated control mice as well as in those mice that received 30 μg DNM in the free form. MBSA alone did not show any significant antitumour activity. In contrast, about 100% suppression of the tumour was achieved when the same dosage of the drug was injected in conjugated form.

To determine the effect of DNM (free or conjugated, on the time course of the tumour growth, the mice injected with J774A.1 tumour cells on day 0 were treated with 30 μg DNM in either the free or conjugated form on days 1, 3, 5 and 7, and the growth of the tumour was observed for 35 days (data not shown). The rate of increase in the tumour mass in PBS-treated control mice paralleled that in the animals that received 30 μg of DNM in the free form. In contrast, the same dose of DNM in the conjugated form caused total suppression of the tumour growth (data not shown). When the tumour-bearing mice were treated on day 1 of tumour implantation with a single bolus of the drug (50 μg/animal), MBSA–DNM prevented further growth of the tumour mass, whereas the tumour weight increased significantly with time in untreated animals or animals treated with an equivalent dosage of free DNM (data not shown).

DISCUSSION

Attempts have been made to use various macromolecular carriers, such as polylysine, DNA,

dextran, and serum albumin, for delivering antitumour drugs [20, 21]. However, these carriers are taken up by the cells primarily through the relatively inefficient processes of fluid phase pinocytosis or non-specific absorptive endocytosis. More recently, various investigators have attempted to exploit cell surface receptor systems for delivering drugs to the tumour cells to overcome the problem of inefficient internalization. Interesting *in vitro* results were obtained using LDL [22] or antireceptor antibodies to peptide hormones [23] as drug carriers. However, since these receptors are also found on most normal cells, it is likely that the drug delivered through such receptors would not be limited exclusively to the tumour cells. Although the LDL receptor-mediated drug delivery has received the most attention thus far [24], the major limitation of this carrier is that only lipophilic drugs can be incorporated into the LDL molecule [25]. 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 the simplicity of preparation, a longer shelf-life, and ease of sterilization and formulation into apyrogenic preparations.

We have shown previously that only the tumour cells of macrophage lineage like J774A.1, P388D1 and IC-21 can recognize and degrade MBSA–DNM, but this drug conjugate is not recognized by cells of non-macrophage origin like L929, EL-4, Bowes melanoma, and CHO [14]. We have also shown that MBSA–DNM can inhibit the proliferation of the receptor-positive cells much more effectively than free DNM while the receptor-negative cells remain unaffected [14].

To gain some understanding of the mechanism by which MBSA–DNM conjugate could elicit antitumour activity, we used J774A.1 cells in culture as an *in vitro* model of macrophage neoplasia and followed the initial binding, internalization and degradation of [^{125}I]MBSA–DNM by these cells. MBSA–DNM bound to the J774A.1 cells with saturation kinetics, indicating that the drug conjugate is recognized by a limited number of binding sites present on the cell surface (Fig. 1a). The high-affinity binding of the drug conjugate to the macrophage tumour cells was inhibited by MBSA but not by DNM, suggesting that the drug conjugate bound to the cells through the receptors that recognized MBSA (Fig. 1b). Subsequently, the bound MBSA–DNM was internalized and degraded to TCA-soluble material (Fig. 2).

The results presented in Fig. 3 demonstrate that MBSA–DNM was cleared rapidly from the circulation and accumulated in the tissues that are rich in macrophages such as liver, spleen, and lung. These results indicate that MBSA–DNM is recognized primarily by the macrophages *in vivo*.

The *in vivo* experiments demonstrated that conjugation of DNM with MBSA resulted in a marked enhancement of the tumouricidal activity of DNM. Thus, 10 μg DNM in the conjugated form caused more than 90% regression of the J774A.1 tumour in BALB/C mice, whereas free DNM at the same dosage did not affect the tumour mass (Fig. 5). The data in Fig. 5 further show that the antitumour efficacy of MBSA–DNM was more pronounced at lower dosages. Thus, 0.8 μg DNM in the conjugated form brought about a 50% reduction in the tumour mass, whereas about 28 μg of free DNM was necessary to achieve the same effect. Time-course experiments (data not shown) demonstrated that administration of MBSA–DNM (4 × 30 μg) in either multiple doses or as a bolus (50 μg) effectively arrested the growth of J774A.1 tumours in mice. In contrast, the same dose of DNM in the free form could not arrest the growth of tumours, and the weight of the tumours resembled that in the untreated control animals. The animals treated with free DNM died within 40–45 days, whereas the mice treated with the drug conjugate remained tumour-free and survived throughout the observed period of 180 days (data not shown). MBSA–DNM effectively caused regression of preformed tumours, whereas free DNM could not suppress the growth of the tumour (Fig. 6). Taken together, our data show that conjugation with MBSA resulted in a major enhancement of the antitumour activity of DNM. MBSA alone did not show any significant antitumour activity. The enhanced antitumour activity of MBSA–DNM probably resulted from the high efficiency uptake of the drug in the conjugated form by the tumour cells through the scavenger receptor-mediated uptake process. However, we have not excluded the possibility that the drug conjugate is taken up by the normal macrophages that migrate to the tumours and act as a slow release depot for the drug. All the animals that received MBSA–DNM remained healthy throughout the experimental period, indicating no apparent toxic effect of the drug conjugate.

In conclusion, our data demonstrated that it is possible to couple an anticancer drug covalently to MBSA without altering the pattern of recognition of MBSA by the macrophage tumour cells. It was also evident that the suppression of the macrophage tumour could be achieved at much lower dosages compared with the free drug by exploiting the scavenger receptor-mediated endocytic pathway *in vivo*. It may also be possible to exploit this receptor system to modulate the macrophage-mediated immune responses where such measures provide therapeutic advantages in various pathological situations by delivering appropriate agents, e.g. in organ transplantation where a reduced immune response is desirable to avoid host/versus graft reaction.

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REFERENCES

1. Young RC, Ozols RF and Meyers CE, The anthracycline antineoplastic drugs. *N Engl J Med* **305**: 139–153, 1981.
2. Buzdar AU, Marcus C, Smith TL and Blumenschein GR, Early and delayed cardiotoxicity of doxorubicin. *Cancer* **55**: 2761–2765, 1985.
3. Tsukada Y, Hurwitz E, Kashi R, Sela M, Hibi N, Hara

A and Hirai H, Chemotherapy by intravenous administration of conjugates of daunomycin with monoclonal and conventional anti-rat α-fetoprotein antibodies. *Proc Natl Acad Sci USA* **79**: 7896–7899, 1982.
4. Forssen EA and Tokes ZA, Improved therapeutic benefits of daunorubicin by entrapment in anionic liposomes. *Cancer Res* **43**: 546–550, 1983.
5. Trouet A, Deprez-de-Campeneere D and De Duve C, Chemotherapy through lysosomes with a DNA–daunorubicin complex. *Nature* [New Biol] **239**: 110–112, 1972.
6. Iwanik MJ, Shaw KV, Ledwith BJ, Yanovich S and Shaw JM, Preparation and interaction of low-density lipoprotein: Daunomycin complex with P388 leukemic cells. *Cancer Res* **44**: 1206–1215, 1984.
7. Schlam J, Basic principles and applications of monoclonal antibodies in the management of carcinomas. *Cancer Res* **46**: 3225–3238, 1986.
8. Basu SK, Receptor-mediated endocytosis of macromolecular conjugates in selective drug delivery. *Biochem Pharmacol* **40**: 1941–1946, 1990.
9. Goldstein JL, Ho YK, Basu SK and Brown MS, Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci USA* **76**: 333–337, 1979.
10. Brown MS, Basu SK, Falck JR, Ho YK and Goldstein JL, The scavenger cell pathway for lipoprotein degradation: Specificity of the binding site that mediates the uptake of negative-charged LDL by macrophages. *J Supramol Struct* **13**: 67–81, 1980.
11. Chaudhuri G, Mukhopadhyay A and Basu SK, Selective delivery of drugs to macrophages through a highly specific receptor: An efficient chemotherapeutic approach against leishmaniasis. *Biochem Pharmacol* **38**: 2995–3002, 1989.
12. Mukhopadhyay A, Chaudhuri G, Arora SK, Sehgal S and Basu SK, Receptor-mediated drug delivery to macrophages in chemotherapy of leishmaniasis. *Science* **244**: 705–707, 1989.
13. Majumdar S and Basu SK, Killing of intracellular *Mycobacterium tuberculosis* by receptor-mediated drug delivery. *Antimicrob Agents Chemother* **35**: 135–140, 1991.
14. Mukhopadhyay A, Mukhopadhyay B, Srivastava RK and Basu SK, Scavenger receptor-mediated delivery of daunomycin elicits selective toxicity towards neoplastic cells of macrophage lineage. *Biochem J* **284**: 237–241, 1992.
15. Cline MJ, The histiocytic malignancies. In: *Cancer Treatment* (Ed. Haskell CM), pp. 952–958. W. B. Saunders, London, 1980.
16. Ghose TI, Blair AH and Kulkarni PN, Preparation of antibody-linked cytotoxic agents. *Methods Enzymol* **93**: 280–333, 1983.
17. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Greke NM, Olson BJ and Klinth DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**: 76–85, 1985.
18. Goldstein JL, Basu SK and Brown MS, Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol* **98**: 241–260, 1983.
19. Geran RI, Greenberg NH, MacDonald MM, Schumacher AM and Abbott BJ, Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chemother Rep* (Part 3) **3**: 51–61, 1972.
20. Ryser HJ-P and Shen W-C, Conjugation of methotrexate to poly(L-lysine) increases drug transport and overcomes drug resistance in cultured cells. *Proc Natl Acad Sci USA* **75**: 3867–3870, 1978.
21. Poznansky MJ, Targeting enzyme albumin conjugates: Examining the magic bullet. *Ann NY Acad Sci* **507**: 211–219, 1987.
22. Lundberg B, Preparation of drug–low density lipoprotein complexes for delivery of antitumoral drugs via the low density lipoprotein pathway. *Cancer Res* **47**: 4105–4108, 1987.
23. Hirota N, Ueda M, Ozawa S, Able O and Shimizu N, Suppression of an epidermal growth factor receptor-hyperproducing tumour by an immunotoxin conjugate of gelonin and a monoclonal epidermal growth factor receptor antibody. *Cancer Res* **49**: 7106–7109, 1989.
24. Bijsterbosch MK and Van Berkel TJC, Native and modified lipoproteins as drug delivery system. *Adv Drug Deliv Rev* **5**: 231–251, 1990.
25. Shaw JM, Shaw KV, Yanovich S, Iwanik M, Futch WS, Rosowsky A and Schook LB, Delivery of lipophilic drugs using lipoproteins. *Ann NY Acad Sci* **507**: 252–271, 1987.