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Enhanced intracellular delivery of doxorubicin by scavenger receptor-mediated endocytosis for preferential killing of histiocytic lymphoma cells in culture

Sunanda Basu, Bratati Mukhopadhyay, Sandip K. Basu*, Amitabha Mukhopadhyay

National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India

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Abstract

A conjugate of the antineoplastic drug doxorubicin (DXR) with maleylated bovine serum albumin (MBSA) was taken up by a human histiocytic lymphoma cell line (U937) through the high efficiency process of scavenger receptor-mediated endocytosis resulting in a sixfold higher intracellular concentration of the drug compared to that obtained when the free drug was administered. Compared to the free drug, the drug conjugate showed significantly higher cytotoxicity towards U937 cells presumably because of intracellular availability of a pharmacologically active form of the drug. The intracellular product released after lysosomal degradation of the drug conjugate was chromatographically identical to free DXR. These findings merit serious consideration in the development of new chemotherapeutic agents for the treatment of histiocytic malignancies.

Key words: Drug delivery; Scavenger receptor; Endocytosis; Histiocytic malignancy; Cancer chemotherapy

1. Introduction

Histiocytic malignancies are aggressive neoplastic diseases which affect the cells of the mononuclear phagocyte system and are usually fatal if untreated. Chemotherapy is the main line of treatment for these disorders. However, reports of the response to single therapeutic modalities in these diseases are fragmentary and no well-documented scientific literature is available. Although promising results have been obtained when treated with combinations of various antitumor drugs [1], the toxicity associated with the chemotherapeutic agents remains the major limitation of the combination therapy.

Doxorubicin (DXR), an anthracycline antibiotic commonly used in the treatment of histiocytic malignancies, causes substantial acute and chronic cardiac toxicity [2], thus limiting the usefulness of the drug. The adverse effects probably arise due to the fact that at therapeutically effective concentrations in the blood, all the cells in the body are exposed to the drug. Such side effects could be minimised if a modality for the administration of the drug could be worked out which would (i) reduce the uptake of the drug through nonspecific diffusion-mediated processes by the nontarget cells, and (ii) selectively deliver the drug only to the target cells at relatively low plasma concentrations. Attempts have been made for selective delivery of cytotoxic agents exclusively to the neoplastic cells using various antibodies, liposomes, DNA and growth factors as carriers [3–6] with varying degrees of success.

As an alternative approach, we have been exploring the possibility of exploiting the cell-type specificity and high efficiency of the scavenger receptor-mediated endocytic pathway [7] for selective delivery of drugs to tumor cells bearing such receptors. Since this receptor system is known to be present primarily on the cells of the macrophage/monocyte lineage, it may be possible to achieve selective delivery of antitumor agents to the neoplastic cells in histiocytic malignancies through these receptors. The scavenger receptors recognize certain polyanionic macromolecules such as maleylated albumin, polyinosinic acid etc. [7]. Previously, we have shown the feasibility of exploiting this receptor system for selective elimination of intracellular parasites in cell culture and/or animal models of leishmaniasis and tuberculosis using various drug conjugates with maleylated bovine serum albumin (MBSA) as a carrier [8–11]. Recently, we have demonstrated that the scavenger receptor-mediated delivery of daunomycin elicits selective toxicity towards murine neoplastic cells of macrophage lineage whereas the receptor negative cells remain unaffected, both in vitro and in vivo [12,13]. As a prelude to determining the feasibility of extending this approach to human malig-

*Corresponding author. Fax: (91) (11) 686 2125.

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; DXR, doxorubicin; FCS, fetal calf serum; MBSA, maleylated BSA; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.

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nancies of macrophage origin, it is important to establish the efficacy of the approach in an appropriate model system.

Accordingly, in the present investigation we have used a well-characterised human cell line U937, derived from a histiocytic malignancy that possesses many features of cells of monocyte/macrophage lineage. Our results establish the superior efficacy of doxorubicin coupled to MBSA (MBSA–DXR) in cessation of DNA synthesis by U937 cells compared to free DXR.

2. Materials and methods

2.1. Materials

Tissue cultures supplies were from Grand Island Biological Co, (Grand Island, NY). Special biochemicals like DXR, BSA, chloroquine, monensin, fucoidin, dextran sulphate, polyinosinic acid, polyguanylic acid, polyadenylic acid, polycytidylic acid, fetuin and heparin were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]DXR hydrochloride and [^{3}H]thymidine were purchased from Amersham International (Amersham, UK). Other reagents used were of analytical grade.

2.2. Cells

U937, a human cell line derived from a patient with histiocytic lymphoma, was obtained from American Type Culture Collection and maintained in medium A (RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and gentamycin (50 μg/ml). These cells were grown at 37°C in a humidified incubator with 5% CO_2 and 95% air atmosphere.

2.3. Maleylation of BSA

BSA was maleylated by reacting the protein with maleic anhydride at pH 8.0 as described by Glazer et al. [14]. Electrophoretic mobilities of the chemically modified protein was tested by 0.7% agarose gel electrophoresis at pH 8.4 [15].

2.4. Conjugation of DXR to MBSA

MBSA was coupled to DXR using glutaraldehyde as described previously [16]. In brief, 6.7 mg of MBSA was mixed with 290 μg of DXR in 1 ml of phosphate-buffered saline (PBS). Glutaraldehyde (0.5%) was added dropwise to the reaction mixture to achieve a final concentration of 0.05% and the reaction mixture was stirred for 30 min at room temperature. The reaction was stopped by adding 50 μl of 1 M lysine and the conjugate was separated from low molecular weight reactants by Sephadex G-50 column chromatography. Void volume fractions containing MBSA-DXR were pooled and subjected to extensive dialysis. The amount of DXR int he conjugate was determined by measuring the absorbance at 495 nm [16]. Protein was estimated by bicinchoninic acid (BCA) reagent [17]. Stoichiometric estimation revealed that the conjugate contained 3–4 mol of drug per mol of protein. [^{14}C]DXR (specific activity, 50 mCi/mmol) was diluted with unlabeled DXR to a specific activity of 133 cpm/ng of DXR and coupled to MBSA under similar reaction conditions. The specific activity the MBSA–[^{14}C]DXR conjugate was 4 cpm/ng protein, i.e. about 30 pg DXR/ng protein.

2.5. Radioiodination of MBSA–DXR

MBSA–DXR was labeled with Na[^{125}I] by the iodine monochloride catalysed reaction as described [18]. More than 99% of the radioactivity was acid precipitable and the specific activity of the conjugate was 186 cpm/ng of protein.

2.6. Uptake and degradation of [^{125}I]BSA–DXR by U937 cells at 37° C

Suspensions containing 1×10^6 U937 cells in 1 ml of medium B (medium A without FCS but with 1 mg/ml of BSA) were placed in each well of 24-well tissue culture plate and incubated in the presence of various concentrations of [^{125}I]MBSA–DXR at 37°C in a humidified incubator with 5% CO_2 and 95% air atmosphere. After 5 h, the cells were collected by centrifugation ($500 \times g$ for 10 min), washed twice with ice-cold PBS containing 1 mg/ml of BSA and twice with PBS. The cells were suspended in 1 ml of 0.1 N NaOH and the amount of radioactivity associated with the cells were determined. The amount of TCA soluble radioactivity accumulated in the medium during the 5 h incubation period was determined in triplicate dishes as described [18].

2.7. Cellular uptake of [^{14}C]DXR and MBSA-[^{14}C]DXR by U937 cells

Suspensions containing 1×10^6 U937 cells along with indicated concentrations of [^{14}C]DXR or MBSA–[^{14}C]DXR in 1 ml of medium B were incubated at 37°C in a humidified incubator with 5% CO_2–95% air atmosphere. After 30 min, the radioactive medium was removed and the cells were washed as described above. The cells from triplicate dishes were dissolved in 1 ml 0.1 N NaOH, aliquots (0.5 ml) of the cell extract were mixed with 5 ml of aqueous scintillation fluid and the radioactivity was measured in a liquid scintillation counter as described before [9]. The protein content of the cell extract was determined by the BCA method. In competition experiments, cells were incubated with 6.8 μg MBSA–[^{14}C]DXR (200 ng DXR) in presence of indicated concentrations of DXR and MBSA–DXR and processed as above.

2.8. HPLC analysis of the intracellular product after degradation of MBSA–[^{14}C]DXR

U937 cells (5×10^8) were incubated in 1 ml of medium B containing 110 ng [^{14}C]DXR in the free or conjugated form at 37°C for 4 h in a humidified incubator with 5% CO_2 and 95% air atmosphere. The cells were washed twice with ice cold PBS containing 1 mg/ml of BSA, followed twice by PBS as above. The drug was extracted into chloroform/methanol (2:1 v/v), evaporated to dryness and reconstituted in 50 μl methanol. The samples from the free drug and the conjugate treated groups were mixed with 25 μg DXR in a total volume of 100 μl and analysed by reverse-phase HPLC on a Nova Pack C18 (150×4 mm) column using TFA-acetonitrile solvent system at a flow rate of 1 ml/min. The elution profile was monitored by absorbance at 495 nm and the radioactivity present in the fractions (1 ml) were determined using a scintillation counter.

2.9. Cytotoxicity assay in vitro

The cytotoxicity of the drug conjugate was determined by the [^{3}H]thymidine incorporation assay [19]. In brief, 1×10^5 cells were incubated overnight in 1 ml medium A at 37°C in a humidified incubator with 5% CO_2 and 95% air atmosphere, washed and incubated in 1 ml medium A containing indicated concentrations of DXR in free or conjugated form for 30 min at 37°C. The cells were then washed thrice with 1 ml of medium A and incubated in 1 ml of drug free medium A at 37°C. After 20 h, the cells received [^{3}H]thymidine (5 μCi/ml). After 3 h, the cells were harvested using cell harvester, washed and radioactivity incorporated by the cells were determined.

3. Results and discussions

To determine the nature of interaction with the drug conjugate, U937 cells were incubated with increasing concentrations of [^{125}I]MBSA–DXR at 37°C for 5 h. Fig. 1a shows that the cell associated radioactivity increased in a saturable fashion. During this period, a part of the added radioactivity was degraded to TCA-soluble material indicating proteolytic degradation of the drug conjugate (Fig. 1b). Half-maximal values for both uptake and degradation of [^{125}I]MBSA–DXR at 37°C were achieved at a concentration of approximately 6 μg of protein/ml. The saturability of both the uptake and the degradation processes becomes even more explicit when the values for the uptake and degradation of [^{125}I]MBSA–DXR in the presence of excess MBSA (300 μg/ml) are substracted from the respective total amounts of [^{125}I]MBSA–DXR

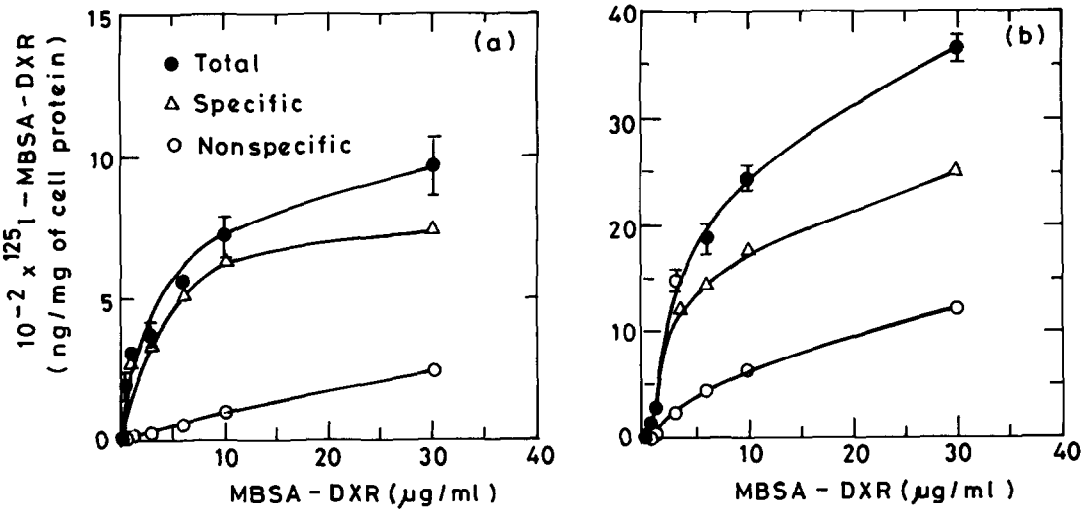

Fig. 1. Concentration dependence of uptake (a) and degradation (b) of [^{125}I]MBSA–DXR by U937 cells at 37°C. Receptor mediated uptake (△) or degradation (△) was obtained by substracting the values for nonsaturable uptake (○) or degradation (○) from the total uptake (●) or degradation (●). Results are expressed as ng of MBSA–DXR associated with the cells or degraded per mg of cell protein (mean ± S.D. for three independent experiments).

associated with the cells or degraded. In order to determine if active lysosomal functions are necessary for the degradation of the drug conjugate, U937 cells were allowed to bind [^{125}I]MBSA–DXR at 4°C for 2 h, washed extensively to remove unbound radioactivity and the cells were incubated in prewarmed (37°C) medium in absence or presence of either monensin (25 μM) or chloroquine (75 μM). After 3 h, the TCA-soluble radioactivity released in the medium of each group of cells was measured. The amounts of [^{125}I]MBSA–DXR degraded per mg of cell protein under these conditions were as follows: untreated control, 449 ± 11; monensin-treated, 292 ± 20; and chloroquine-treated, 160 ± 3.6. Thus, lysosomal inhibitors monensin and chloroquine reduced the degradation of [^{125}I]MBSA–DXR to 65% and 35% of that by untreated control cells, respectively, indicating that the radiolabeled MBSA-DXR was internalized and degraded in the cellular lysosomes in absence of the inhibitors. The data in Fig. 1 also show that the amount of TCA-soluble radioactivity accumulated in the medium after 5 h was 2–3 times higher than the cell-associated radioactivity. The facts that lysomotropic agents inhibit the degradation of the drug conjugate, taken together with the data in Fig. 1 showing saturability of the uptake and degradation of the drug conjugate, suggest that the binding, internalization and subsequent lysosomal degradation of the drug conjugate are mediated through a limited number of binding sites which are probably recycled and participate in multiple rounds of ligand delivery into the cells. These conclusions are in consonance with our previously published results with similar drug conjugates [8–13] as well as those of others [7,18].

Fig. 2 compares the cellular uptake of the free conjugated DXR as a function of DXR concentration in the medium. When the cells were incubated for 30 min in presence of [^{14}C]DXR (300 ng/ml) either in free or conjugated form, the cell-associated DXR amounts were about 16 ng/mg and 100 ng/mg of cell protein, respectively. Thus the administration of the drug in the conjugated form resulted in a sixfold higher uptake compared to the free drug in the same time interval. Presence of an excess of MBSA in the medium effectively prevented the uptake of MBSA–[^{14}C]DXR, whereas free DXR did not have any significant effect, indicating that the drug conjugate was recognised by the macrophages through the MBSA moiety (data not shown).

In order to determine whether the enhanced uptake of MBSA–DXR was mediated by the scavenger receptors, cells were incubated at 37°C with 6 μg/ml [^{125}I]MBSA–DXR along with various negatively charged compounds (6 μg/ml). The results presented in Table 1 show that negatively charged compounds like MBSA, fucoidin, dextran sulphate, polyguanylic acid and polyinosinic acid that are reported to be recognised by the scavenger receptor system, effectively competed for the degradation of the [^{125}I]MBSA–DXR. In contrast, compounds not recognised by these receptors, viz. fetuin, polyadenylic acid, polycytidylic acid and heparin were much less effective. These data indicate that the drug conjugate is taken up by the U937 cells through the scavenger receptor system.

In an attempt to identify the pharmacologically active species of the drug released after intracellular degradation of the drug conjugate, U937 cells were incubated with MBSA–[^{14}C]DXR or free [^{14}C]DXR for 5 h, and extracted with $CHCl_3/CH_3OH$. The materials extracted in the solvent were analysed by HPLC. The data pre-

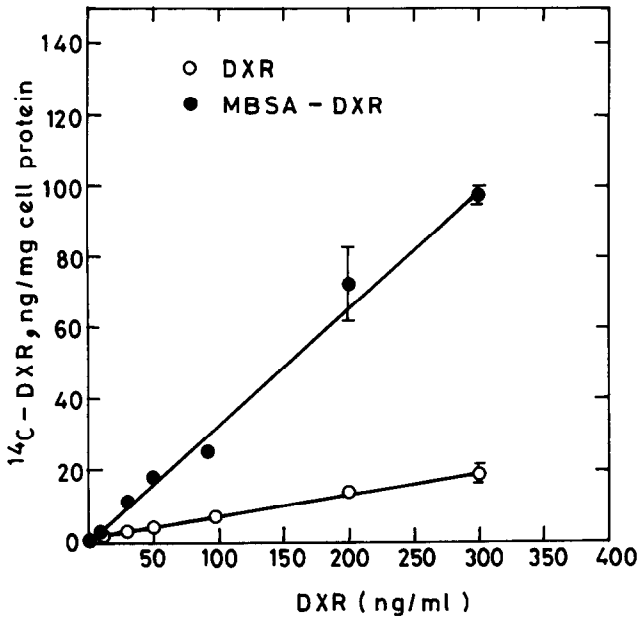

Fig. 2. Uptake of different concentrations [^{14}C]DXR in free or conjugated form by U937 cells at 37°C. Results are expressed as ng of [^{14}C]DXR taken up per mg of cell protein (mean ± S.D. for three independent experiments).

sented in the Fig. 3 show that the material obtained from the cells treated with MBSA–[^{14}C]DXR shows a single major peak of radioactivity with the same retention time as the authentic unlabelled DXR used as standard. The radioactivity from the [^{14}C]DXR treated cell samples was also eluted from the column at the same position as that of authentic DXR standard (data not shown) indicating that incubation with the cells did not alter the mobility of DXR. These results suggest that the product released after lysosomal degradation of the conjugate is chromatographically identical with the free drug.

To determine the cytotoxic efficacy of the intracellular product released after lysosomal degradation of the drug conjugate, we have compared the effects of the free and conjugated DXR on incorporation of [^{3}H]thymidine by U937 cells. The data presented in Fig. 4 show that free DXR (0.3 μM) inhibited about 50% of the [^{3}H]thymidine incorporation compared with the untreated control cells. In contrast, the same concentration of DXR reduced [^{3}H]thymidine incorporation by over 90% in the receptor-bearing U937 cells. The superior cytotoxic activity of the drug conjugate was more pronounced at lower concentrations. Thus, 0.03 μM of the drug in the conjugated form reduced 50% of the [^{3}H]thymidine incorporation compared with the untreated control, whereas 0.3 μM of the free drug was needed to achieve the same percentage of inhibition. We have previously shown that in receptor-negative Bowes melanoma cells only 10% of the [^{3}H]thymidine incorporation was inhibited by the same concentration of the drug in the conjugated form (data not shown; [12]). Time-course studies (data not shown) revealed that the rate of [^{3}H]thymidine incorporation by the U937 cells treated with free DXR (0.2 μM) over a 24 h experimental period paralleled that in the untreated control cells, whereas, the same concentration of DXR in the conjugated form arrested the ability of these cells to incorporate [^{3}H]thymidine within 2 h of exposure.

The data presented in this paper show that the conjugation of DXR with MBSA significantly increased the cytotoxic activity of the drug to the scavenger receptor bearing U937 cells (Fig. 4). The enhanced antitumor efficacy of the drug conjugate is likely to be due to high affinity binding of the carrier to the cell surface receptors followed by rapid internalization and subsequent degradation of the carrier in the lysosomes leading to enhanced intracellular release of a pharmacologically active species of the drug. Presumably, the rapid recycling of the scavenger receptors for multiple rounds of ligand delivery contribute to the building up of high intracellular concentrations of the drug in the target cells when MBSA–DXR is used (Fig. 2). Furthermore, the HPLC analysis of the intracellular product generated after degradation of the drug conjugate shows that it is likely to be the native DXR (Fig. 3).

In this study, we have used MBSA as a homing device to deliver an antitumor agent (DXR) specifically to human neoplastic cells of macrophage origin exploiting the scavenger receptor mediated endocytic pathway. The

Table 1
Degradation of [^{125}I]MBSA–DXR by U937 cells: competition by negatively charged macromolecules

Effective competitor	[^{125}I]MBSA–DXR degraded (ng/mg of cellular protein)	Ineffective competitor	[^{125}I]MBSA–DXR degraded (ng/mg of cellular protein)
MBSA	896 ± 80	Fetuin	1302 ± 42
Fucoidin	593 ± 46	Heparin	1060 ± 68
Dextran sulphate	506 ± 2.8	Polyadenylic acid	1346 ± 24
Polyinosinic acid	386 ± 19	Polycytidylic acid	1020 ± 11
Polyguanylic acid	283 ± 4.5		

Competition of degradation of [^{125}I]MBSA–DXR (6 μg/ml) by U937 cells at 37°C was measured in absence or presence of same concentrations (6 μg/ml) of various polyanionic macromolecules. In absence of any competing compound, U937 cells degraded 1613 ng of [^{125}I]MBSA–DXR per mg of cellular protein during 5 h incubation at 37°C.

approach of receptor mediated drug delivery to cancer cells using various receptor systems for lipoproteins, growth factors, etc. has been reported previously [3–6]. But the ubiquitous distribution of these receptors on normal cells is the major drawback for selective delivery of the drug specifically to the tumor cells. Nevertheless, interesting in vitro results have been obtained using low-density lipoprotein as a carrier [20]. However, the major limitation of this carrier is that only lipophilic drugs can be incorporated into the LDL molecules. Moreover, lipoproteins are complex molecules with limited stability which are difficult to formulate into stable pharmacological preparations. In contrast, MBSA as a drug carrier has certain advantages over the existing ones because of the simplicity of preparation, longer shelf life, and ease of sterilization and formulation into apyrogenic preparations.

These studies, in conjunction with the earlier reports [12,13] from this laboratory demonstrating superior therapeutic potential of MBSA-daunomycin in suppressing the growth of solid tumors of macrophage lineage in BALB/C mice, raise the interesting prospect of using this mode of chemotherapeutic approach against neoplastic diseases like histiocytic malignancies which are difficult to control by conventional chemotherapy. Moreover, this receptor system also offers the possibility of manipulating the metabolism of macrophages in order to suppress or enhance immune responses.

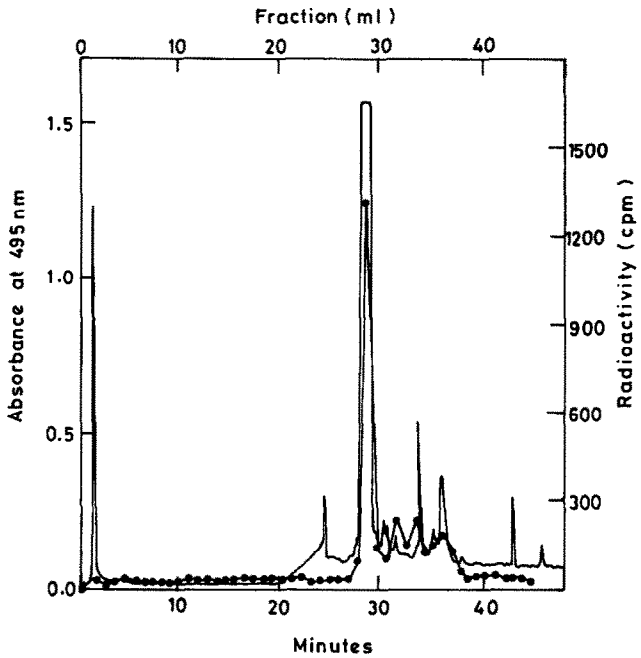

Fig. 3. Identification of the intracellular product released after degradation of the drug conjugate by U937 cells. The sample was taken in methanol, mixed with 25 μg doxorubicin in a total volume of 100 μl and injected to the column (Nova Pack C 18) equilibrated with solvent A (0.1% TFA in water). The run was continued with solvent A for 10 min, then a gradient of solvent B (80% acetonitrile containing 0.1% TFA) was employed as follows: 0 to 30% solvent B in 40 min and 30% to 100% in the next 10 min. Elution profile was monitored as described in section 2. Absorbance, (–); Radioactivity, (●).

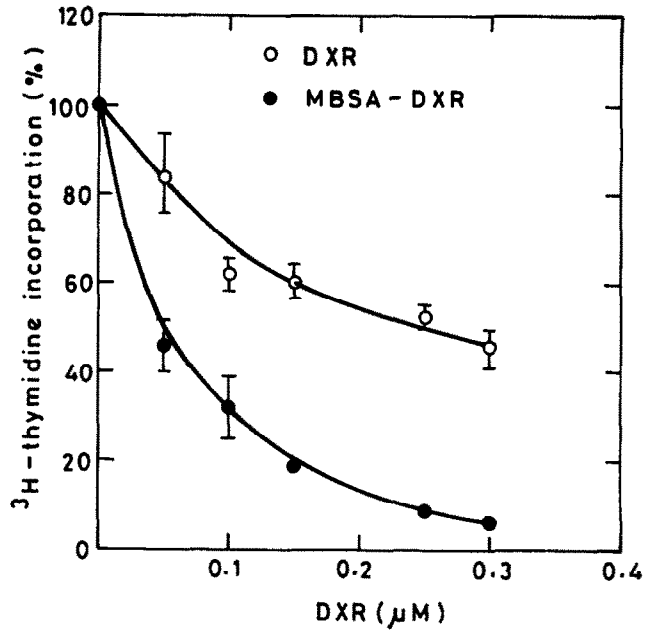

Fig. 4. Uptake of [^{3}H]thymidine by U937 cells in presence of DXR in free or in conjugated form. The results are expressed as percentages of the uptake of [^{3}H]thymidine by untreated cells (mean ± S.D. for three independent experiments).

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