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THE SELECTIVITY OF ACTION OF ALKYLATING AGENTS AND DRUG RESISTANCE—III

THE UPTAKE AND DEGRADATION OF ALKYLATING DRUGS BY YOSHIDA ASCITES SARCOMA CELLS *IN VITRO*

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Abstract—The uptake and utilisation of Myleran (busulphan), Alkeran (melphalan) and Leukeran (chlorambucil) by drug-sensitive and -resistant strains of a Yoshida ascites sarcoma have been studied *in vitro*. No differences were detected in the ability of either cell strain to incorporate these agents. The pattern of uptake, and subsequent hydrolysis, of melphalan and Myleran was identical in both cell strains.

Hydrolysis of the mustard group of chlorambucil in resistant cells occurred at a rate comparable to that of an aqueous solution of the drug, while in sensitive cells it was 2·5 times slower. Rupture of the aromatic ring of chlorambucil occurred in both cell strains, though this reaction was restricted in -sensitive compared with -resistant cells. These combined effects resulted in the maintenance of a higher level of "active" chlorambucil in the sensitive cells, and possibly contributed to its selective lethal effects.

There is no evidence of permeability differences between the two cell strains which might account for the failure of the resistant strain to respond to chemotherapy with alkylating agents.

A PROBLEM encountered frequently in the application of alkylating agents to the chemotherapy of neoplastic disease is the development of drug resistance.[1, 2] It has not been established whether this is due to the gradual emergence of an altered population of tumour cells which are refractory to treatment, or alternatively to modification of the tumour cells through long-term contact with the drugs.

The Yoshida ascites sarcoma[3] responds to treatment with alkylating agents, and provides a useful experimental tumour with which to investigate certain aspects of this problem. A form of the neoplasm, refractory to treatment, and cross-resistant to a number of alkylating agents, has been obtained following long-term exposure to melphalan (Alkeran).[4] Several biochemical properties of these two tumour strains, possibly associated with drug reactivity or inactivation, have already been examined.[5]

When alkylating agents were administered to rats carrying the drug-sensitive Yoshida ascites sarcoma, an accumulation of DNA, RNA, protein, glutathione, and certain glutathione-metabolising enzymes occurred in the tumour cells: comparable changes were not seen in resistant tumour cells following drug treatment. This failure of chemotherapy to produce identical biochemical changes in both cell strains may have been due to differences in drug uptake, and the present communication describes the results of experiments which investigated this possibility. The uptake of

chlorambucil (Leukeran), melphalan (Alkeran), and busulphan (Myleran) by the two cell strains *in vitro*, and the subsequent fates of the three drugs have been investigated.

MATERIALS AND METHODS

Leukeran (chlorambucil) $(ClCH_2CH_2)_2N.C_6H_4(CH_2)_3COOH$, Myleran (busulphan) $CH_3SO_2O(CH_2)_4OSO_2CH_3$ and Alkeran (melphalan) $(ClCH_2CH_2)_2N.C_6H_6$ CH_2 CH NH_2 COOH were synthesised in the Chester Beatty Research Institute. Other chemicals were purchased from Hopkin and Williams Ltd., or B.D.H. Ltd., AnalaR grades being used where available.

Animals. Full details of the animal experimentation and tumour transplantation techniques have been given previously.[6, 7] Animals were killed by cervical dislocation on the sixth day after tumour transplantation. The peritoneal contents were aspirated with 5 ml 0·3% w/v sodium chloride (containing 0·05% trisodium EDTA) pH 7·3, and any erythrocytes present were removed by selective osmotic lysis according to the procedure described by Walford.[9]

Cell suspensions. $1·2 \times 10^9$ cells were suspended in 11·0 ml 0·9% w/v sodium chloride solution (containing 0·5% w/v trisodium EDTA) and the pH adjusted to 7·3. Drug-treated cell suspensions were prepared in duplicate, together with a solvent-treated cell suspension, and a control composed of the suspending medium and drug only. Drug solution, or solvent alone, was added to the mixtures in 1 ml volumes. Incubations were carried out in a metabolic shaker (Gallenkamp) at 37°. Details of the drugs and solvents used are listed in Table 1.

TABLE 1. DRUG CONCENTRATIONS AND SOLVENTS USED

Drug	Solvent	Drug concn (mg/ml)
Chlorambucil	Ethanolic-HCl/ propylene glycol*	5·4
Melphalan	Ethanolic-HCl/ propylene glycol*	5·4
Myleran	Dimethylsulphoxide	13·5

1·0 ml of drug solution or solvent was added to 11 ml of cell suspension.

* The drug was dissolved in one volume of 2% w/v HCl in ethanol and diluted with nine volumes of phosphate/propylene glycol solution (prepared by dissolving 20 g dipotassium hydrogen phosphate and 450 ml propylene glycol in water and diluting to 1 l.

Drug uptake was followed by withdrawing 2 ml aliquots of cell suspension at measured time intervals after drug addition. Cells were removed by centrifugation at 500 *g* (0°), the supernatant diluted 10-fold in an appropriate extractant (see below), and the precipitated protein removed by centrifugation at 500 *g*, 0°. The packed cells were resuspended to a volume of 6 ml in the same extractant and subjected to a 20 kc/sec sonic oscillation for 2 min (MSE Ultrasonic disintegrator): precipitated protein was removed by centrifugation as before. The "total drug" content of these extracts, determined from the optical density at 258 mμ, provided a measure of all those modifications of chlorambucil or melphalan which contained an intact benzene

ring. The residual alkylating ability ("active" drug) of each of the three agents was estimated by a modification of the colorimetric procedure of Epstein.[10]

Estimation of chlorambucil. 99·5% ethanol was used as the drug extractant/protein precipitant.

"Total" drug determinations were made after a 10-fold dilution of the extracts in 99·5% ethanol. After E^1_{258} had been measured, the "total" chlorambucil content of the extracts was determined by reference to a standard curve prepared under the same experimental conditions.

The residual alkylating ability ("active" drug) was determined by adding 2 ml 2% w/v *p*-nitrobenzylpyridine (PNBP) in ethylene glycol to 1 ml of the ethanol extract and heating at 95° in a stoppered tube for 10 min. After cooling in ice, 2 ml 50% w/v triethylamine solution in acetone was added, the contents mixed thoroughly, and E^1_{565} measured within 2 min. The concentration of "active" chlorambucil was estimated by referring to a standard curve prepared under the same experimental conditions.

Estimation of melphalan. 0·1 N sodium ethoxide in ethanol was used as the drug extractant/protein precipitant (melphalan is only slightly soluble in ethanol alone). This soln was used freshly prepared and was standardised by titration against 3% w/v HCl in ethanol, using bromothymol blue as indicator. The protein-free extracts were neutralised with 3% w/v HCl in ethanol, 0·2 ml water per ml of solution being added to prevent salt precipitation.

"Total" drug. The neutralised protein-free extract was diluted 10-fold with 3% w/v HCl in ethanol, E^1_{258} measured, and compared with a standard curve prepared under the same experimental conditions.

Residual alkylating ability ("active" drug). Aliquots of the neutralised protein-free extracts were analysed by the procedure used for "active" chlorambucil, incubation at 95° being extended to 60 min.

Estimation of Myleran (residual alkylating ability "active" drug). 99·5% ethanol was used as the drug extractant. The assay procedure was identical to that for "active" chlorambucil, except that the period of incubation at 95° was extended to 30 min.

RESULTS

The pattern of uptake of chlorambucil, melphalan and Myleran is summarised in Figs. 1, 2, 3, respectively: in each case uptake occurred only during the first 5 min of the incubation period. Both sensitive and resistant cells accumulated equal quantities of the agents, an equivalent loss of drug occurring from the suspending medium in each case. For chlorambucil (Fig. 1) and melphalan (Fig. 2), where both "total" and "active" drug measurements were made, it was apparent that no hydrolysis of the mustard groups of these two compounds occurred during the period of uptake. While both cell strains took up approximately two thirds of the chlorambucil present initially in the suspending medium (Fig. 1), only one third of the content of melphalan (Fig. 2) or Myleran (Fig. 3) was removed. In the case of the latter two drugs, the rate of hydrolysis occurring in the incubation medium was comparable to that in control media devoid of cells. The levels of melphalan or Myleran retained by the cells declined at comparable rates in either cell strain.

In contrast, certain differences *were* apparent when the behaviour of chlorambucil was compared in suspensions of sensitive and resistant cells. No hydrolysis of this

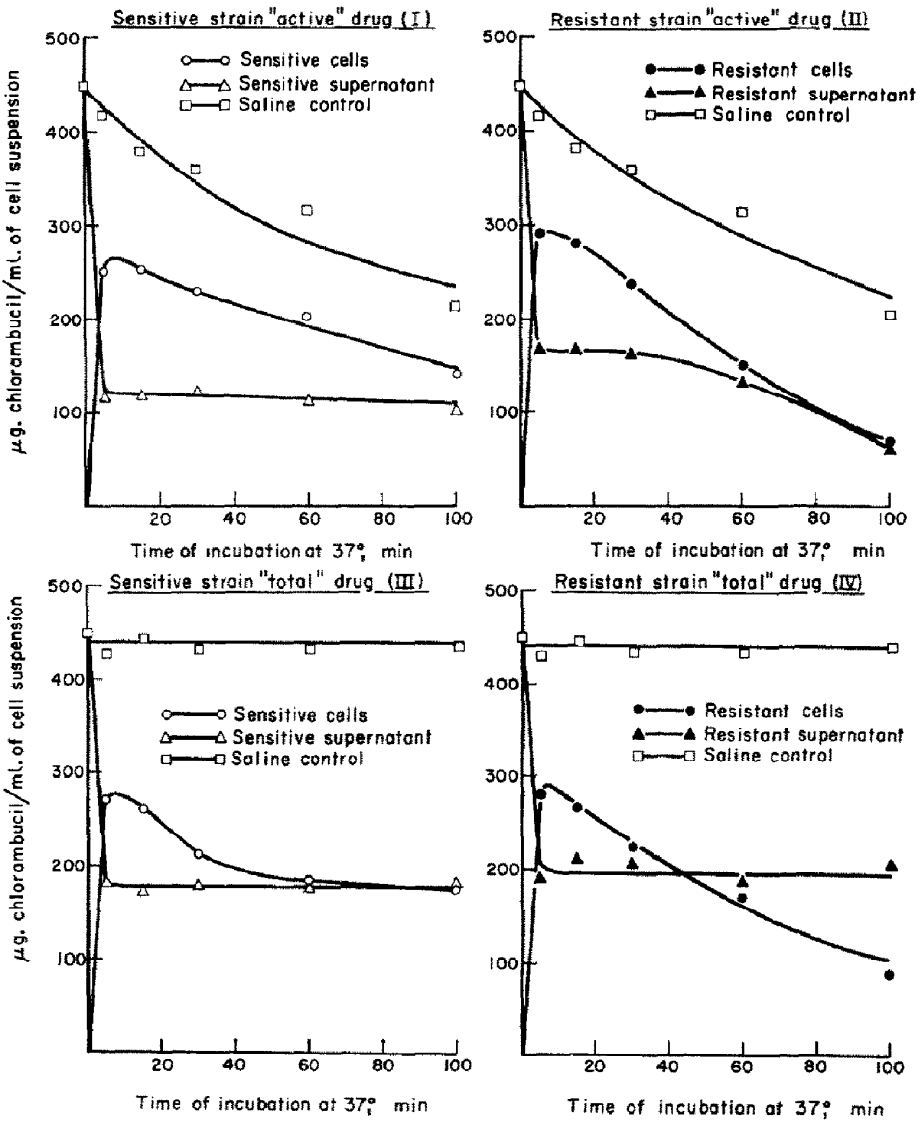

FIG. 1. Distribution of chlorambucil in "total" and "active" forms, between drug-sensitive and resistant Yoshida ascites cells and their incubation media.

●—● resistant cells; ▲—▲ resistant supernatant

○—○ sensitive cells; △—△ sensitive supernatant

□—□ saline control.

Each point represents the mean of eight determinations. The overall scatter about any point $\not>$ 10 per cent.

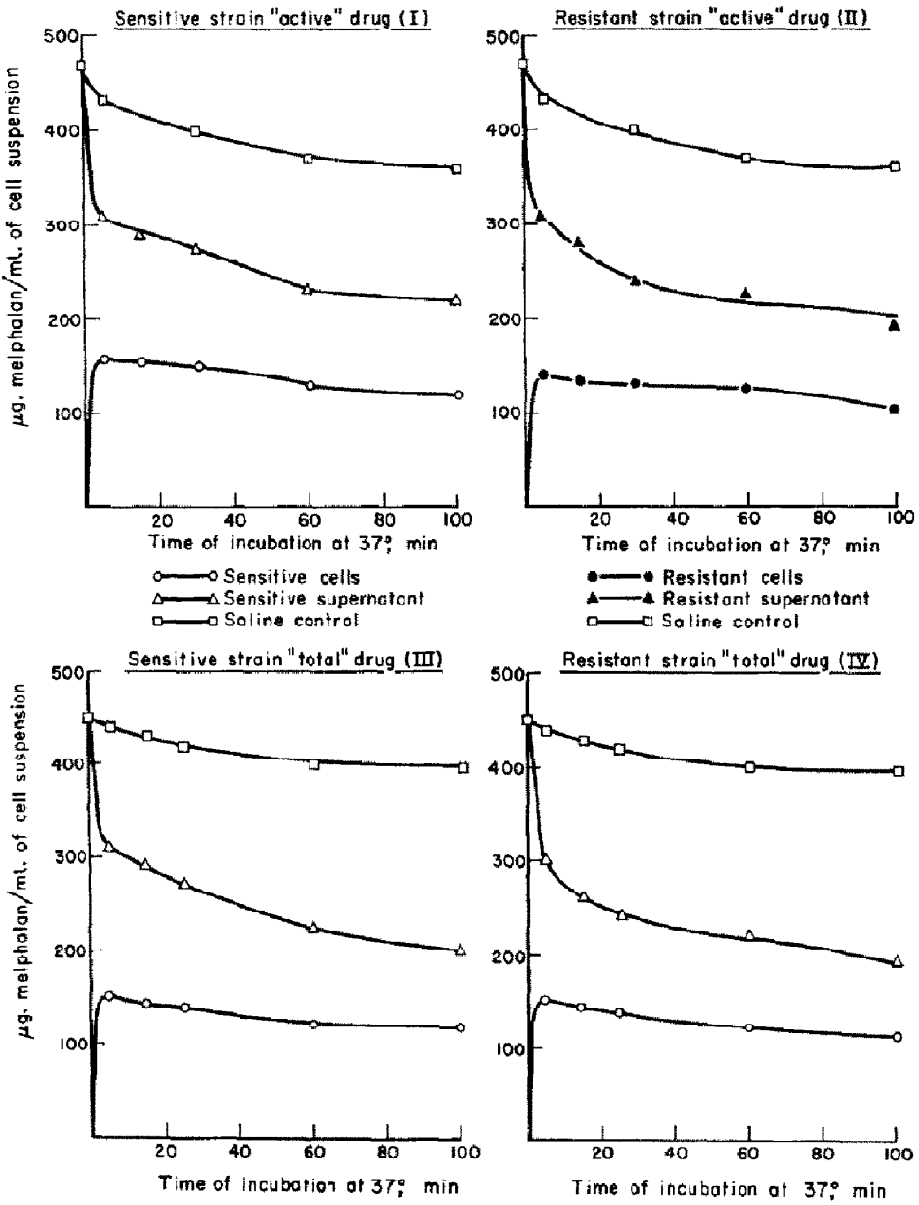

FIG. 2. Distribution of melphalan in "total" and "active" forms, between drug-sensitive and -resistant Yoshida ascites cells and their incubation media.

●—● resistant cells; ▲—▲ resistant supernatant

○—○ sensitive cells; △—△ sensitive supernatant

□—□ saline control.

Each point represents the mean of eight determinations. The overall scatter about any point ≯ 10 per cent.

drug occurred at any time during the incubation period in suspension media containing sensitive cells, while in the presence of resistant cells hydrolysis commenced after 40 min [Fig. 1(i), (ii)]. Furthermore, the residual alkylating ability of chlorambucil retained by resistant cells declined at approximately 2·5 times the rate of that in sensitive cells [Fig. 1(i), (ii)]. Additional differences between the two cell strains were revealed when their "total" contents of chlorambucil were compared [Fig. 1(iii), (iv)]. The ultraviolet absorption at 258 mμ in extracts of resistant cells declined linearly

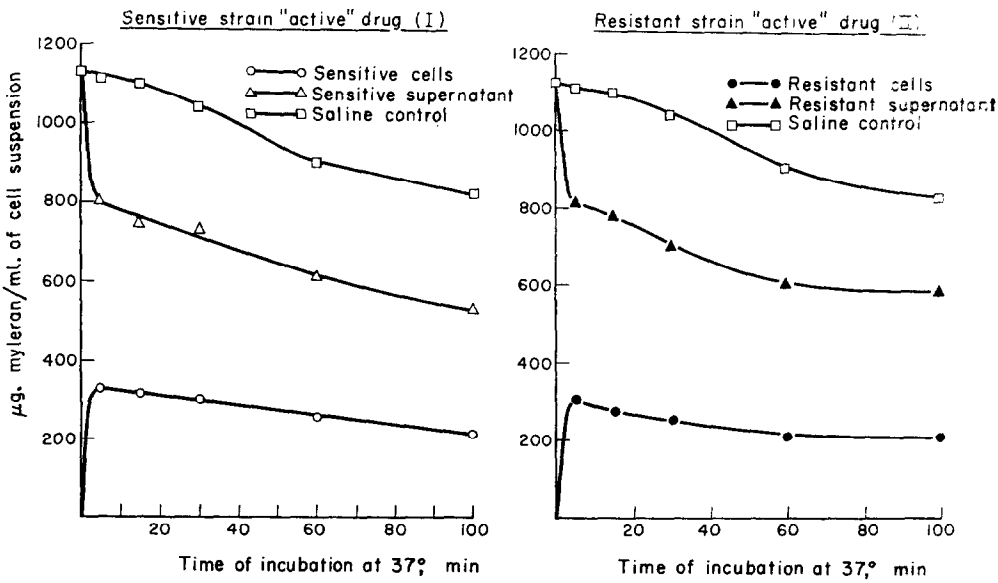

FIG. 3. Distribution of Myleran in the "active" form, between drug-sensitive and -resistant Yoshida ascites cells and their incubation media.

●—● resistant cells: ▲—▲ resistant supernatant

○—○ sensitive cells; △—△ sensitive supernatant

□—□ saline control.

Each point represents the mean of eight determinations. The overall scatter about any point ⩾ 10 per cent.

throughout the period of incubation, and although this effect was also seen in sensitive cells, it was limited to the first 30 min of incubation. These effects were not seen when melphalan was substituted for chlorambucil [Fig. 2(iii), (iv)]. This loss of u.v. absorbing material associated with the cells was not due to covalent binding of chlorambucil to protein. When the protein precipitate was dissolved in alkali, no increase in u.v. absorption (attributable to bound drug) was detected.

The absolute amount of chlorambucil taken up by the cells was proportional to its concentration in the suspension medium. In Fig. 4 it can be seen that a second dose of drug added to suspensions of sensitive and resistant cells is accumulated and degraded in the manner already described.

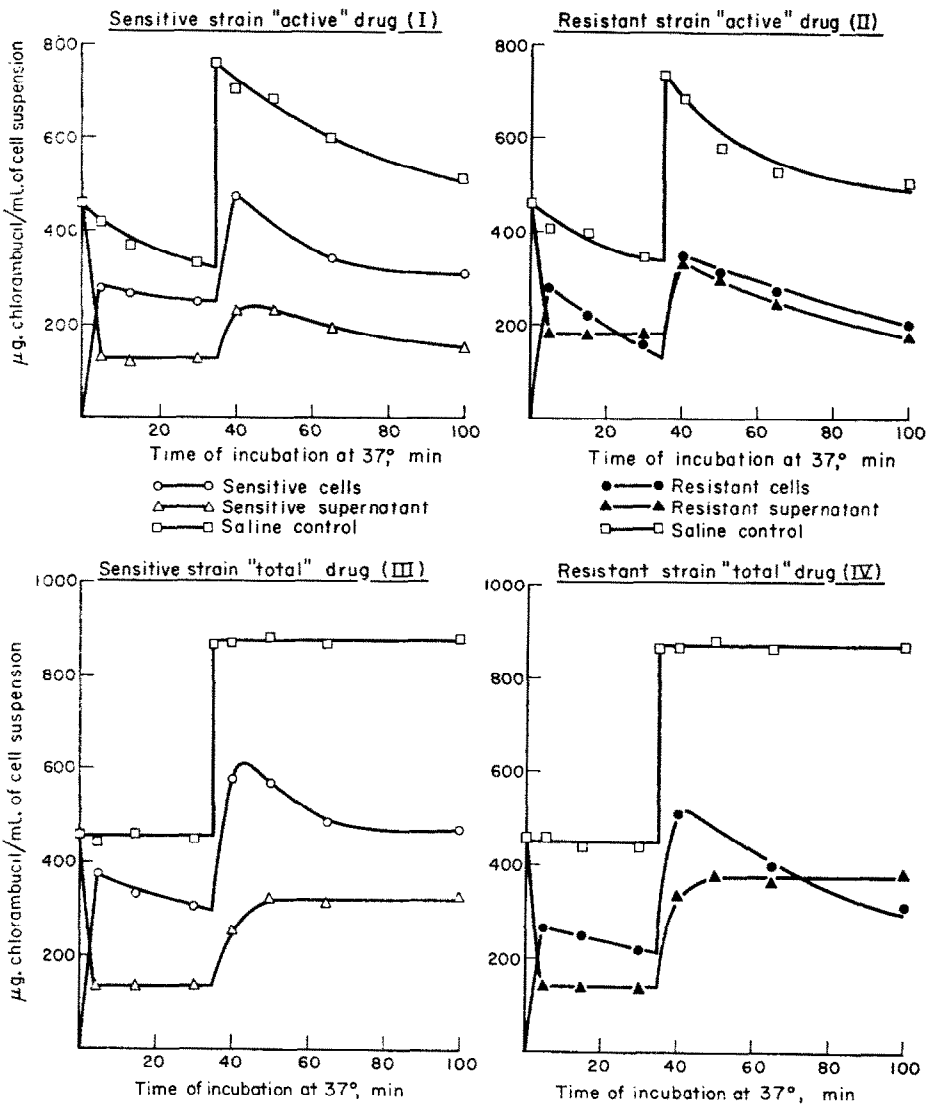

FIG. 4. Distribution of chlorambucil in "total" and "active" forms, between drug-sensitive and -resistant Yoshida ascites cells and their incubation media. Drug was administered at zero time followed by a second dose 30 min later.

●—● resistant cells; ▲—▲ resistant supernatant

○—○ sensitive cells; △—△ sensitive supernatant

□—□ saline control.

Each point represents the mean of eight determinations. The overall scatter about any point ± 10 per cent.

DISCUSSION

These results indicate that both cell strains incorporated a constant fraction of the drug to which they were exposed, uptake being restricted to the first 5 min of incubation. The agents were incorporated in the "active" form, and both cell strains incorporated an identical quantity of each drug.

Only in the case of chlorambucil were differences in drug utilisation apparent between the two cell strains. In this case, drug remaining in the suspending medium of sensitive cells was not hydrolysed, while in the presence of resistant cells hydrolysis started after approximately 40 min of incubation. Hydrolysis of chlorambucil retained by the resistant cells was comparable to that of a saline "control", while in sensitive cells hydrolysis occurred at less than 0·4 times this figure. The aromatic ring of the drug was degraded in both cell types, though the extent of this reaction was limited in sensitive cells. Effectively, the decreased rate of drug hydrolysis, coupled with the limited degradation of the aromatic ring of the drug, leads to the maintenance of a higher level of active compound in the sensitive cells compared with that in resistant cells.

It has been shown that alkylating drugs do not induce identical biochemical changes in tumour cells *in vivo*,[6, 7] while in the present study a different *in vitro* utilisation of these agents has been demonstrated. These differences in reactivity may be associated with factors contributing to the clinical selectivity of alkylating agents (i.e. the use of chlorambucil in the treatment of chronic lymphocytic leukaemia, of Myleran in treating chronic myeloid leukaemia, and melphalan for the control of myelomatoses[11, 12, 13]). Sanderson and co-workers have also observed the differing biological reactivities of a number of alkylating agents.[14] However, it remains a surprising finding that drug-resistant cells possess an enhanced ability both to degrade and to hydrolyse chlorambucil, while the closely related compound melphalan was utilised similarly by both cell strains.

Linford has described the hydrolysis of chlorambucil, and its various condensation reactions with serum proteins as a possible source of therapeutic "waste".[15] The metabolic scission of the aromatic ring of this compound has not been detected previously.

The relative abilities of drug-sensitive and -resistant tumour cell strains to incorporate alkylating agents have also been studied by Gáti and Körös,[16] and by Novikova and co-workers.[17] The former authors ascribed the resistance of a strain of NK/lymphoma (to degranol) to an enhanced ability of the resistant cells to bind the drug. The latter authors isolated ten times more sarcolysin bound to the DNA of a resistant transplantable sarcoma 45 than could be detected in the DNA of a sensitive tumour (both tumours being carried bilaterally in the same animal).

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