Department of Physics and Biophysics[1]; Department of Chemistry and Biochemistry[2], Medical Institute of Thracian University, Stara Zagora, Bulgaria

Influence of some DNA-alkylating drugs on thermal stability, acid and osmotic resistance of the membrane of whole human erythrocytes and their ghosts

I. T. IVANOV[1] and V. GADJEVA[2]

Human erythrocytes and their resealed ghosts were alkylated under identical conditions using three groups of alkylating antitumor agents: mustards, triazenes and chloroethyl nitrosoureas. Osmotic fragility, acid resistance and thermal stability of membranes were changed only in alkylated ghosts in proportion to the concentration of the alkylating agent. All the alkylating agents decreased acid resistance in ghosts. The clinically used drugs sarcolysine, dacarbazine and lomustine all decreased osmotic fragility and thermal stability of ghost membranes depending on their lipophilicity. DM-COOH did not decrease osmotic fragility and thermal stability of ghost membranes, while NEM increased thermal stability of membranes. The preliminary but not subsequent treatment of ghosts with DM-COOH fully abolished the alkylation-induced thermal labilization of ghost membrane proteins while NEM had a partial effect only. The present study gives direct evidence that alkylating agents, having a high therapeutic activity against malignant growth, bind covalently to proteins of cellular membranes.

1. Introduction

Chloroethylnitrosoureas, triazenes and nitrogen mustards are compounds known as alkylating agents and are used clinically for the treatment of a wide variety of human and animal neoplasms. Single agents of particular interest include triazene 5-(3,3-dimethyltriazene-1-yl)-imidazole-4-carboxamide (dacarbazine, DTIC) with greatest antitumor effect against methastatic melanoma [1, 2], nitrosourea, 1-cyclohexyl-3-(2-chloroethyl)-L-nitrosourea (lomustine, CCNU) [2, 3] and nitrogen mustards such as sarcolysine *p*-[*bis*-(2-chloroethyl)-amino)-DL-phenylalanine [4] for the treatment of lymphomas, melanomas, gliomas and few solid tumors. Recently, a promising combination of interferon-α plus fourdrug chemotherapy (dacarbazine, vincristine, bleomycine and lomustine), for methastatic melanoma has been reported to exhibit a remarkably high response rate of 62% [5]. All of these highly reactive anticancer drugs decompose spontaneously under physiological conditions to give electrophiles which produce alkylation and/or interstrand cross-linking of DNA and proteins [6, 7]. Nitrosoureas carbamoylate thiol groups of glutathione causing changes in cellular glutathione content [8]. Glutathione and endogenous thiols have been considered to play a significant role in the radiation protection of cells [9] and also as possible indicators of chemosensitivity [10].

Due to its pharmacological importance the binding of different antitumor DNA-modifying agents, including alkylating agents to cellular proteins has attracted a great deal of attention. Thulin et al. [11] have found that mustards readily bind to hemoglobin as a significant number of adducts to N-terminal valines in hemoglobin could be found after exposure of red cell hemolysate to nitrogen mustard in vitro. Moreover, Black et al. [12] have isolated alkylated glutathione and globin from blood preincubated with sulphur mustard, following lysis of erythrocytes. N-terminal valine, on both the alfa and beta chains, and histidine residues were identified as the key site for interaction of sulphur mustard with hemoglobin.

Studies using EPR spectroscopy and preincubation experiments with *N*-ethylmaleimide (NEM) revealed that the spin labelled nitrosourea l-chloroethyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea, a compound with a high antitumor activity, penetrated the erythrocyte membranes within three minutes and bound predominantly to two classes of SH groups of membrane proteins that have strong and week mobilities of the nitroxyl moiety, respectively [13]. Studies carried out with some alkylating antitumor drugs have shown that they bind to cellular membranes as well [14, 15]. Considering their structure, the binding of these drugs to cellular membranes and their transfer through them is, however, still not well understood.

In the present study we report our investigations on the osmotic fragility, acid resistance and thermal stability of membranes of human erythrocytes before and after treatment with a number of compounds representing the main groups of alkylating agents. Whole cells and isolated membranes, both alkylated under identical conditions, were used for comparative study. A possible relationship between the chemical structures of the alkylating agents and their binding to membrane proteins of erythrocyte ghosts has been discussed. Human erythrocytes are frequently employed in such investigations and they appear to be a useful cellular model. Knowledge of the interaction of these drugs with erythrocytes could also be important in view of the involvement of erythrocytes in the transport of drugs with blood circulation and delivery of drugs to target tissues.

2. Investigations, results and discussion

Mustards (sarcolysine, CEAS, CEH and BEH), triazenes (DTIC and DM-COOH) and chloroethyl nitrosoureas (CCNU and TNU) were used in the present study. When suspended in acidic media, intact erythrocytes and their one-step resealed ghosts lyse by a mechanism that includes the following steps: a) transfer of acid into cytosole, which is the rate limiting step; b) oxidation of hemoglobin, coupled to release of free radicals and c) oxidative stress on cellular membranes resulting in egress of hemoglobin [21]. We show the time course of HCl − incuded hemolysis of human erythrocytes (Fig. 1 A) and their ghosts (Fig. 1B) subjected to preliminary alkylation with CEAS. The alkylation was carried out under identical conditions for cells and ghosts by incubating them at 20 °C,

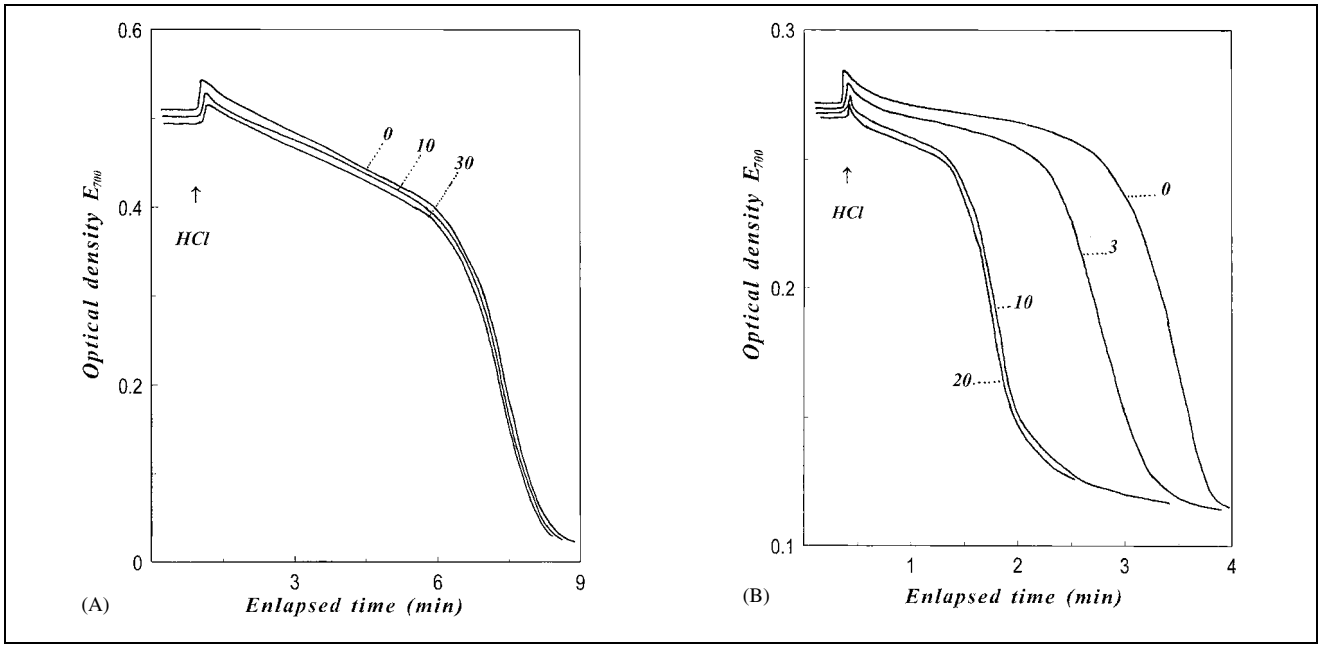

Fig. 1. HCl – induced hemolysis of human erythrocytes (A) and their one-step resealed ghosts (B) as affected by pre-alkylation. The cells and ghosts were incubated at 20 °C in NaCl-saline containing 2.0 mM CEAS, 5 mM phosphate buffer, pH 7.0, hematocrit 0.20. After a time interval, indicated in min on the panel, a portion of the suspension was withdrawn and mixed with 1.8 ml NaCl saline, dilution between 50 and 200 times. 5 min later, 20 µl HCl was added and the induced hemolysis was followed photometrically at 700 nm under constant stirring

hematocrit 20%, in buffered saline, pH 7.0, that contained 2 mM of the agent. At the times indicated, portions of the resulting suspension were withdrawn and diluted in saline (about 100 times dilution) to test acid resistance. After 5 min to allow the removal of unbound agent from alkylated cells and ghosts, hemolysis was started by addition of a hydrochloric acid load. Control experiments proved that the amount of unbound alkylating agent in the hemolytic media did not affect the acid resistance of cells and ghosts (not shown).

Compared to intact cells, the acid resistance of alkylated cells was practically unchanged by alkylation that continued for up to 30 min (Fig. 1A). During the same alkylation period, acid resistance of ghosts was, however, strongly reduced (Fig. 1B) indicating increased rate of the transfer of acid into the cytosole. These findings indicated that in contrast to the membranes of whole cells, the membranes of ghosts were hardly affected by alkylation with CEAS. On the time scale, the reduction in acid resistance of ghosts took place between 3 and 8 min alkylation and was not changed by further incubation (Fig. 1B). This suggests that the entire quantity of agent was taken up by ghosts within the given time interval. Similar results were obtained using the other mustard triazene and nitrosourea alkylating agents (not shown).

The osmotic fragility of membranes of erythrocytes corresponds to the osmoactivity of media where cells overcritically swell and lyse. It depends on both the expandability of membranes and cell surface area to cell volume ratio. The osmotic resistance was assayed in whole cells and

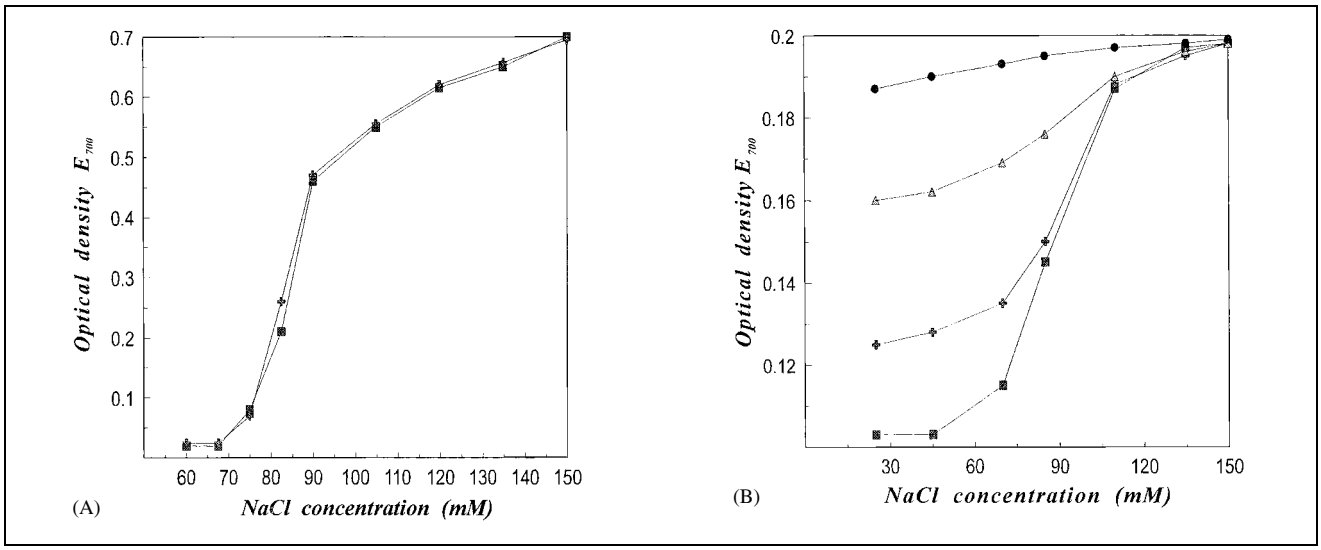

Fig. 2.: Osmotic fragility of whole erythrocytes (A) and one-step resealed ghosts (B) as affected by alkylation with different concentrations of CEAS. Packed cells 0.1 ml were suspended in 0.4 ml NaCl saline which contained 5 mM phosphate buffer, pH 7.0, with 2 mM CEAS (+) or without CEAS (■). Likewise, 0.1 ml packed ghosts were suspended in 0.4 ml NaCl saline which contained 5 mM phosphate buffer, pH 7.0, and the indicated concentrations of CEAS: 2 mM – (○): 0.6 mM – (△); 0.2 mM – (+) and 0 – (□) as control. After 15 min alkylation, the alkylated cells and ghosts were transferred into hypotonic medium of NaCl, indicated on the abscissa, and optical density readings were taken in 2 min

resealed ghosts before and after their alkylation with CEAS carried out under identical conditions as described above. According to the decrease in light scattering (Fig. 2A), the intact cells and ghosts swelled at mild hypotonicity (150–100 mM sodium chloride and lysed in a media with sodium chloride concentration about 85 mM. The alkylation did not change the osmotic fragility of cells at all (Fig. 2A) but strongly modified the osmotic response of ghosts at hypotonicity (Fig. 2B). This finding indicated that the ionic permeability barrier of the membranes was preserved in cells and strongly perturbed in ghosts after incubation in the same alkylation medium. Similar disturbance of the permeability barrier of ghost membranes was obtained with all the mustards employed plus dacarbazine and CCNU but not with DM-COOH and tyrosine nitrosourea (not shown).

Curve 1 in Fig. 3 shows the derivative thermogram of the conductivity of a suspension which contained resealed one-step ghosts under an outward gradient of ionic concentration. The thermogram had precisely the same form when ghosts replaced whole erythrocytes (not shown). As has been determined previously [29] the peaks centered at 52 °C and 66 °C correspond to the denaturation of spectrin and conformation change in intrinsic proteins, respectively. Thus, the maximum temperatures T_m of those peaks expressed the thermal stability of peripheral and intrinsic groups of membrane proteins, respectively. In order to study the impact of alkylation on the structural stability of membranes, similar thermograms were obtained with suspensions that contained whole cells or resealed ghosts alkylated with CEAS. Prior to heating, the cells (ghosts) were alkylated for 15 min as above, isolated, washed in excess saline, and heated in a low-salt isotonic medium. Compared to intact cells, the maximum temperatures T_m of both peaks were practically unchanged in alkylated whole cells but were strongly decreased in alkylated ghosts (Fig. 3). Similar results were obtained with other alkylating agents. The reduction in T_m could be used as a measure of the thermal destabilisation of membrane proteins related to their alkylation.

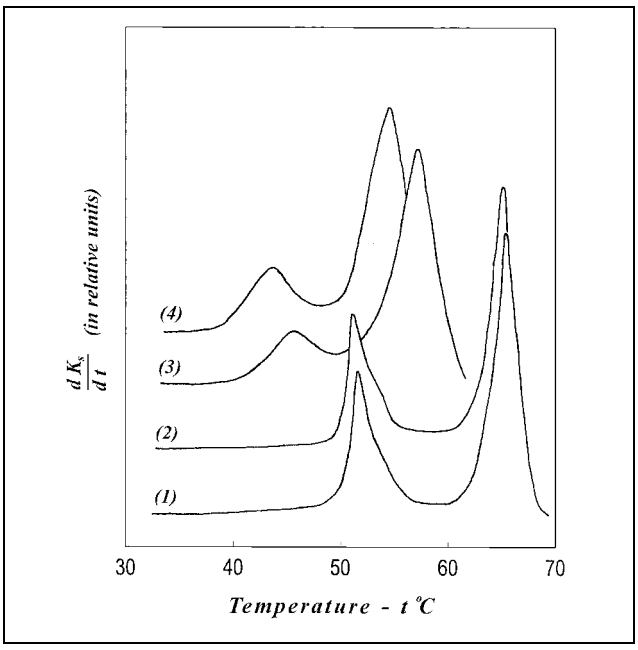

Fig. 3: Derivative thermogram of the electrical conductivity of suspensions containing one-step resealed ghosts (1-control), alkylated erythrocytes (2), alkylated one-step resealed ghosts (3) and alkylated two-step resealed ghosts (4). The first and second peaks indicate threshold changes in suspension conductivity related to thermal denaturation of spectrin and the integral proteins, respectively (Ivanov, 1997). Alkylation of cells and ghosts was conducted at 20 °C in NaCl saline that contained 1 mM CEAS, hematocrit 0.15. After 15 min, they were withdrawn, washed free of CEAS and suspended in isotonic 50 mM NaCl/sucrose medium. The suspension was further heated recording the first derivative of its conductivity on a chart. The frequency of the current, hematocrit, and heating rate were 1 kHz, 0.07 and 2.0 °C/min, respectively

The destabilisation effect of taurine mustard on membrane proteins, clearly varied in ghosts depending on their hemoglobin content. It was minimal in one-step ghosts and gradually increased with two- and three-step ghosts with further reduced hemoglobin content (Fig. 3). When alkylation of hemoglobin-free ghosts was conducted in a medium that also contained albumin, the resulting destabilisation of membrane proteins was increasingly inhibited as the concentration of albumin increased (not shown). Apparently, both hemoglobin encapsulated within the cellular membranes and external albumin decreased the destabilisation effect of alkylation on membrane proteins.

Thus, using the tests of thermal stability, acid resistance and osmotic fragility, the alkylation of whole cells and their ghosts, carried out under identical conditions, altered the membranes mainly in ghosts. The ghost membranes became structurally modified as shown by the decrease in their thermal stability and disturbance of the permeability barrier for ions and acid. This effect could be related to the higher degree of alkylation of membrane proteins in ghosts as compared to that in whole cells. Given that mustards bind readily to hemoglobin in hemolysate [11] and in whole blood [12] this outcome could be due to the presence of hemoglobin and other non-membrane proteins during the alkylation procedure. Because of their overwhelming amount, the non-membrane proteins of whole cells may be able to bind most of the alkylating agent resulting in a very low level of alkylation of membrane proteins. This explanation is consistent with the results that the alkylation-produced destabilisation in ghosts became stronger as the hemoglobin content decreased and also became weaker in the presence of external albumin.

These findings imply that before binding to membrane proteins, the alkylating agents rapidly penetrated the erythrocyte membrane during the alkylation period, which is consistent with previous data obtained with spin-labelled chloroethyl-nitroso urea [12, 13]. It is important to find out how the alkylating agents were transferred through the membranes of erythrocytes. Ethanolamine, nitrogen mustard (HN_2), nitrogen halfmustard and choline are all believed to be transported by the choline permease in the membranes of yeast cells [17], He-La cells and rat thymocytes [18] and rat nerve cells [19]. Choline permease is, however, practically absent in human erythrocytes [20] and could not be the route of penetration into cytosole for these drugs.

For some of the agents employed, the possible pathways of penetration into cells could be elucidated through the determination of hemolysis. Cells suspended in an unbuffered medium began to lyse following the introduction of CEAS (Fig. 4A) which is a strong acid. The hemolysis was rapid and dose-dependent in intact cells and markedly slower and less rigorous in cells with the anion channel blocked by DIDS. This finding demonstrated that CEAS, like hydrochloric acid, induced hemolysis after entering the cytosole through the anion channel of membranes [21]. The sulphate moiety of CEAS possibly enabled this agent to be recognised and trasferred through the band 3 protein of the erythrocyte membrane thus playing the role

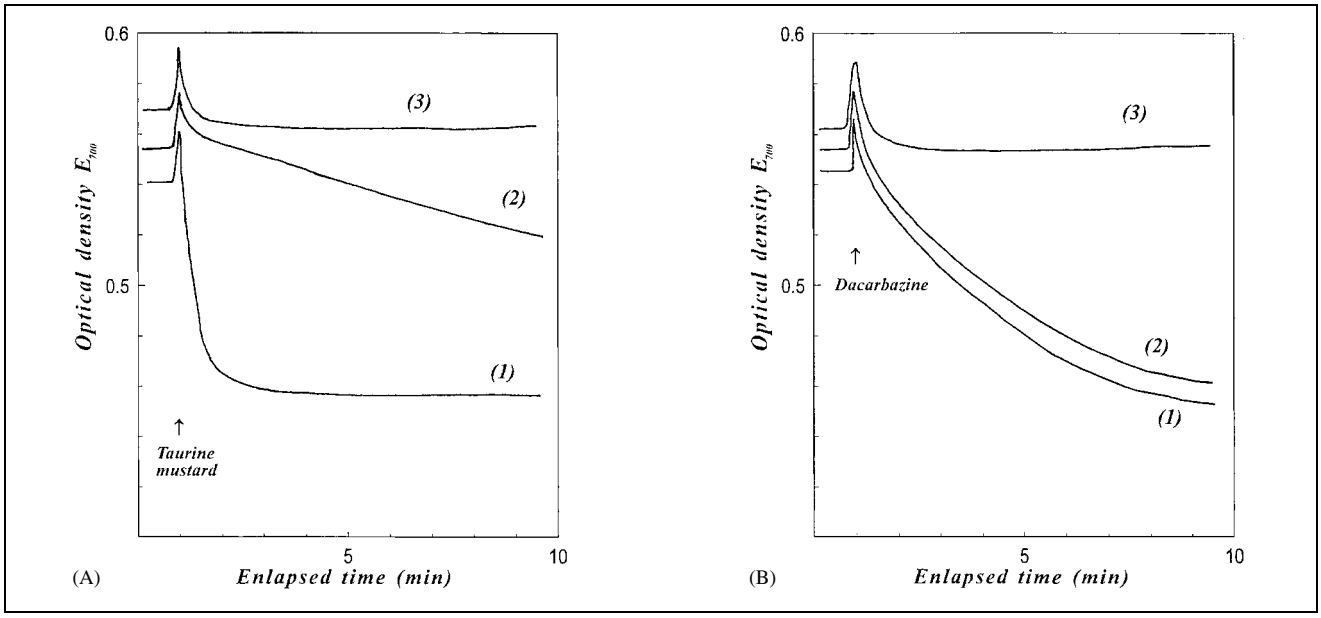

Fig. 4.: Time-profile of the light scattering of erythrocyte suspension after addition of taurine mustard (A) or dacarbazine (B). The suspension contained unbuffered NaCl-saline and intact cells (1) or cells with anion channel inhibited by DIDS (2) or NaCl-saline buffered by 30 mM Triss. HCl, pH 7.0 and intact cells (3). At the time indicated by the arrow the alkylating agent was injected under constant stirring and changes in optical density OD_{700} were recorded on a chart. The decrease in OD_{700} indicates reduced light scattering as a result of swelling and hemolysis

of carrier for the agent. In unbuffered medium, hemolysis was also induced by acidic dacarbazine, which apparently entered cytosole by routes other than the anion channel as hemolysis did not depend on the preliminary inhibition of anion transport (Fig. 4B). According to the molecular structure of dacarbazine, the undissociated form of this agent could have sufficient lipophilicity to pass through the lipid bilayer of membranes thus introducing the agent into cytosole by a protonophore mechanism. Neither agent caused hemolysis in buffered neutral medium (Fig. 4). Moreover, other alkylating agents (Tyrosine-nitrosourea, CCNU), which are not dissociable, did not cause hemolysis even in unbuffered medium (not shown). This data possibly indicated that the hemolytic activity of alkylating agents was strongly potentiated at acidic conditions. Based on the results with CEAS, the low pH in cytosole was mainly responsible for the increased cytotoxicity of alkylation. It is known that at low pH DTIC decomposes into a powerful electrophilic agent 5-diazoimidazole-4-carboxamide (diazo − AIC) that is able to bind to cellular proteins [7].

Byrne et al. [22] have found that alkylation of cystein-containing proteins with CEAS generated significant amounts of covalently cross-linked protein dimers. This was due to preferential alkylation of protein SH-groups as the cross-linking did not occur in model proteins which have no cystein residues. In this study, the ghost membranes were pre-alkylated with *N*-ethylmaleimide (NEM), a specific reagent for SH-groups in proteins, in order to assess the tendency of ghost membrane SH-groups for subsequent alkylation with the agents studied. When the alkylation was carried out with NEM alone, the T_m of the first and second peaks were increased (Table 1) which demonstrated increased stability of membrane proteins. Moreover, in ghosts pre-alkylated with NEM (up to 10 mM), subsequent alkylation with CEAS (1.6 mM) gave a destabilisation effect reduced by 30% compared to that produced by alkylation with CEAS alone (Table 1). When the second alkylation was carried out with sarcolysine, the destabilisation effect was reduced more markedly (Table 1). These results proved that only a part of the labilization

Table 1: Impact of different alkylating agents on the denaturation temperatures of ghost membrane proteins, spectrin and intrinsic proteins

Membrane preparation	T_{m1} (°C)	T_{m2} (°C)
Control (intact) membranes	52.0	66.0
Alkylated with 4 mM NEM	52.9	66.4
Alkylated with DM-COOH	52.3	66.2
Alkylated with 1.6 mM CEAS	38.0	46.5
Alkylated with 1.6 mM CEAS and pre-alkylated with 10 mM NEM	44.0	57.0
Alkylated with 1.6 mM CEAS and pre-alkylated with 1.6 mM DM-COOH	51.0	65.0
Alkylated with 1.6 mM DM-COOH and pre-alkylated with 1.6 mM CEAS	39.0	48.0

The SD from the mean value was about 0.3 °C in most cases

effect was due to the binding of mustards to SH-groups of membrane proteins. When pre-alkylation was carried out with DM-COOH (1 mM), subsequent alkylation with either sarcolysine or CEAS at a concentration less than that of DM-COOH, did not produce any destabilisation of membrane proteins (Table 1). Such destabilisation was apparent, however, if the molar concentration of mustard was greater than that of DM-COOH (Table 1). Thus, mustard-induced destabilization of ghost membranes was partially reduced by preliminary treatment with NEM and totally eliminated by DM-COOH. However, DM-COOH, at any concentration, did not affect mustard-induced destabilization in ghosts, when treatment with it was subsequent to the alkylation of the ghosts with mustards (Table 1). The latter results possibly indicated that both DM-COOH and the mustard competed to bind the same types of chemical groups, although the binding of DM-COOH conferred stability on proteins while binding of mustards caused structural destabilisation.

In one-step ghosts, alkylated with CEAS, the T_m of both peaks demonstrated different but linear dependence on the concentration of the agent (Fig. 5). Hemoglobin-free ghosts were also used to quantify the destabilisation of membrane proteins produced by various alkylating agents.

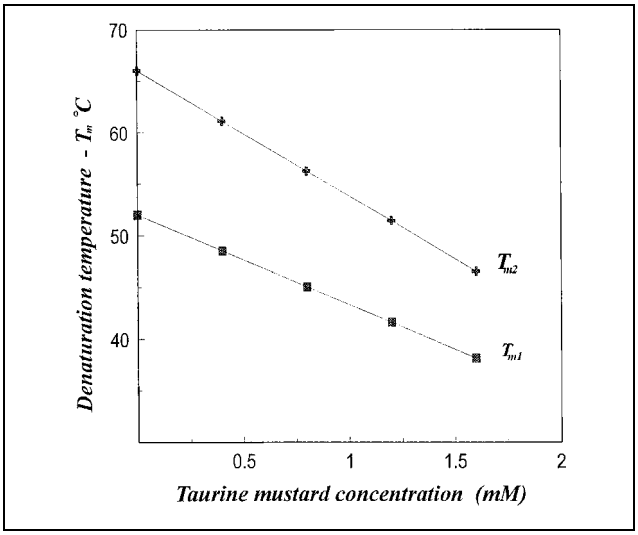

Fig. 5: Thermal destabilisation of erythrocyte ghost membrane proteins by alkylation. One-step ghosts were alkylated with different concentrations of CEAS, washed, and heated under outward ion concentration gradient as described for Figure 3. The denaturation temperature of spectrin (T_{m1}) and integral proteins (T_{m2}) was determined by the top temperature of the peaks described for Fig. 3

The potency of the agent to thermally destabilise membrane proteins was defined as the reciprocal of that concentration of the agent that produced a 10 °C decrease in the top temperature of the respective peak. Table 2 shows the potencies of different alkylating agents to destabilise spectrin (denoted as P_1) and the anion channel (P_2). For different mustards, these potencies clearly depended on both the carrier and halogen atom. The destabilisation potency of CEAS and CNNU was greater than that of less hydrophobic agents such as H2-N-mustards and sarcolysine. The destabilisation potency of Br-mustard was greater than that of Cl-mustard (Table 2). This could possibly be explained by the different partitioning of the agent between the membrane and water phase depending on the lipophilicity of the agent. Considering the agents as amphiphilic molecules, the destabilisation potencies increased with increasing hydrophobicity of their nonpolar part. Clearly, both spectrin and the integral proteins were destabilised more efficiently by mustards with greater hydrophobicity of their nonpolar moiety.

These results are essentially in agreement with the established activity-lipophilicity relationship for some alkylating antitumor drugs. According to Sosnovsky et al. [23], the most hydrophilic compounds should exhibit the highest antitumor activity suggesting hydrophilic sites were targeted. The above results indicated that the destabilisation effect on membranes was mainly produced by the molecules of the agent that resided in the hydrophobic sites of membranes. In addition, Table 2 shows that the P_2/P_1 ratio of destabilisation potencies was significantly greater in more hydrophobic alkylating agents. In comparison to the peripheral protein spectrin, the anion channel is more exposed to the lipid phase and, thus it is more susceptible to labilization by agents that partition predominantly into the lipid milieu. Thus, the agents with greater lipophilicity: CCNU, sulphur mustard and Br-mustard, produced greater labilization on the anion channel compared to spectrin. For all the alkylating agents employed, it could be assumed that the rate of their transfer across the membranes apparently prevailed over the rate of their binding to proteins. Consequently, during the initial stage of alkylation period, the alkylating agent could be expected to achieve a near equilibrium distribution and partitioning across the cellular membranes allowing different destabilisation of membrane proteins.

Although all of the alkylating agents used reduced the acid resistance of resealed ghosts, they were not equally potent in their effect on the barrier and thermal stability of membranes. We showed that those alkylating agents (mustards plus dacarbazine and CCNU) which were able to disturb the permeability barrier at the same time decreased the thermal, and/or structural, stability of ghost membranes. By contrast, the alkylation of membrane proteins with NEM, DM-COOH and tyrosine nitrosourea was not accompanied by barrier disruption and structural destabilisation of ghost membranes. Comparing the structural differences between DM-COOH and DTIC, and between CCNU and TNU, the different efficacy of these agents in disturbing membranes may be explained by their different carrier moiety. This information could shed light on the role that the carrier moiety plays in the cytotoxic effects of alkylating agents.

Table 2: Potency P (Mol^{-1}) of different alkylating agents for thermal destabilisation of the proteins of erythrocyte membrane, spectrin (P_1) and intrinsic proteins (P_2)

Alkylating drug	P_1 (Mol^{-1})	P_2 (Mol^{-1})	P2/P1
H_2N-mustard (Cl)	180 ± 10	240 ± 13	1.3
H_2N-mustard (Br)	290 ± 15	420 ± 20	1.45
$HSO_3-CH_2-CH_2-N$ mustard (CEAS)	870 ± 35	1200 ± 50	1.40
Phenylalanine-N-mustard (sarcolysin)	1250 ± 50	340 ± 18	0.27
HSO_3-N-mustard	ND	ND	1.70
DTIC (dacarbazine)-triazene	230 ± 13	240 ± 13	1.05
DM-COOH-triazene	0	0	ND
Tyrosine-nitrosourea	$< 60 \pm 10$	$< 60 \pm 10$	ND
CCNU	200 ± 12	760 ± 35	3.8

Mean value ± SD of three different determinations is shown

3. Experimental

3.1. Alkylating agents

The antitumor drugs dacarbazine (DTIC), lomustine (CCNU) and sarcolysine were kindly donated by Bristol-Myer Squibb Co. (Wallingford, CT, USA). The nitrogen mustards *N,N′-bis*(2-chloroethyl)aminoethane sulfonic acid (CEAS), *bis*-(2-chloroethyl)-hydrazine (CEH) and *bis*-(2-bromoethyl)-hydrazine (BEH) were purchased from Aldrich Chemical Company. 3,3-Dimethyl-(4-carboxyphenyl)-triazene (DM-COOH) and 1-(2-chloroethyl)-3-[(3′-p-hydroxyphenyl)methyl-propanoil]-1-nitrosourea (TNU) were synthesised by procedures previously reported [24, 25]. The triazenes dacarbazine and DM-COOH were used as 100 mM stock solutions in DMSO. To achieve sufficient solubility, the stock solution of DM-COOH was diluted in medium containing 5 mM Triss-HCl buffer, pH 7.4. DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid) was purchased from Sigma Chemical Co, St. Louis, MO, USA. The alkylating activity of the agents used was determined spectrophotometrically using 4-(*n*-nitrobenzyl)-pyridine (NBP) reagent [25].

3.2. Erythrocytes and erythrocyte membranes

Red blood cells were isolated from freshly drawn human blood of healthy donors and washed three times in NaCl-saline. One-step resealed ghosts were obtained by the procedure of Bodemann and Passow [26]. Erythrocytes were lysed in a hypotonic medium that contained 5 mM phosphate buffer, pH 7.4, 0.5 mM $MgCl_2$ and 0.2 mM EGTA. The packed cells to lysing medium volume ratio was 1:20. Following the restoration of isotonicity to 150 mM NaCl and resealing of membranes at 37 °C for 20 min, the ghosts were separated by centrifugation. The hemoglobin content of the ghosts was further reduced in two- and three-step resealed ghosts prepared by repeating this procedure with one-step resealed ghosts.

Prior to use, the resealing of the membranes was verified by two independent methods sensitive to perturbation of the permeability barrier. The first consisted of obtaining the Boyle van't Hof relation of ghost volume with

inverse of osmotic pressure [27] which is linear providing the permeability barrier exists. The other was based on recording the derivative thermogram of the conductivity of a suspension that contained ghosts under an outward ion concentration gradient [28]. With resealed ghosts, one is able to record a pair of sharp isothermic peaks at 52 °C and 66 °C related to spectrin denaturation and thermal pore formation in membranes, respectively (Fig. 3, curve 1). The second peak is absent in non-resealed ghosts as they cannot maintain the imposed gradient.

3.3. Determination of the peak temperature T_m that induces thermal denaturation of membrane proteins

During transient heating of a suspension of cells and ghosts, the conductivity of the suspension, measured at 1 kHz, underwent sharp changes about 52 °C and 66 °C [28]. These changes were conveniently detected as positive peaks by recording the first derivative of the conductivity on a chart (Fig. 3, curve 1). These threshold changes in electric properties of membranes revealed thermally-induced conformation changes in two different portions of membrane proteins, peripheral and intrinsic proteins, respectively [29]. The 52 °C peak corresponded to a decrease in dielectric polarizability of membranes [30] associated with the denaturation of spectrin [29] which takes place at 49.5 °C [31]. The 66 °C peak was due to the formation of pores in membranes at 62 °C and related collapse of the ion concentration gradient [28]. These peaks were shifted rightward because of the heating rate applied. At lower heating rates, the maximum temperature of each peak was decreased extrapolating towards the denaturation temperature (49.5 °C or 62 °C) of the respective event (not shown). The method is assumed not to be sensitive to changes in the shape of cells during heating.

Intact cells or resealed ghosts (content usually 150 mM NaCl) were suspended (hematocrit 0.07) in an isotonic 50 mM NaCl/sucrose medium, thus imposing an outward ion concentration gradient across the membranes. The suspension was heated with constant heating rate and the output signal U_s of a conductometer was fed into a differentiating amplifier, the output voltage U of which was recorded (derivative conductivity thermogram).

The dependence of U_s on the temperature, T, is closely linear: $U_s = U_{so} \cdot (1 + K_s \cdot \Delta T)$, where K_s is the temperature coefficient of U_s. When T increases at a steady-rate, V, U could be expressed as $U = 1/(R \cdot C) \cdot dU_s/dt = 1/(R \cdot C)$. $U_{so} \cdot K_s \cdot V$, where $1/(R \cdot C)$ is the amplification coefficient. During the heating, only K_s is allowed to change, which sensed the possible change in suspension conductivity. Time differentiation was applied in order to compensate for the strong Boltzmann dependence of the suspension conductivity on the temperature. At steady-rate heating, the thermogram appeared as a horizontal line, unless the suspension conductivity increased, which caused a sharp peak around the inducing temperature T_m. At 2.0 °C/min heating rate, the reproducibility of T_m was within $+/-0.3$ °C.

3.4. Acid hemolysis of alkylated cells and ghosts

Low pH hemolysis of cells and ghosts was induced by adding 20 µl HCl-load to a continuously stirred 1.8 ml suspension. The final pH was determined by a pH-meter at simulative conditions. Hemolysis was followed by recording the changes in optical density at 700 nm (OD_{700}) on a chart [32]. At this wavelength the light absorption of hemoglobin is nil and the OD_{700} measured can be attributed mainly to the light scattering of the cells which remain intact. The acid resistance of cells is defined as the time elapsed after acidification of the suspension, during which 50% of cells lyse as measured by the reduction in optical density. Generally, the scattering of light from an erythrocyte suspension is proportional to the concentration of cells provided their volume remains constant. At a constant cell concentration, the change of volume can also contribute, swelling decreasing and shrinkage increasing the scattering and consequently the optical density of the suspension [33].

3.5. Osmotic test of alkylated whole cells and one-step resealed ghosts

Stock suspension of cells or ghosts was prepared by suspensing 0.1 ml packed cells or ghosts in 0.4 ml NaCl saline which contained 5 mM phosphate buffer with or without alkylating agent, at pH 7.0. After 15 min to allow alkylation, the stock suspension of ghosts was diluted in 1.2 ml NaCl-containing medium to make it hypotonic. Likewise, a small amount of the alkylated cells was transferred into 1.8 ml hypotonic medium of NaCl. The optical density of the suspension obtained was measured at 700 nm (OD_{700}) in 2 min. OD_{700} depended on the final NaCl concentration of the medium as a proportion of the cells and ghosts swelled or lysed according to their osmotic fragility.

3.6. Inhibition of the anion channel of erythrocyte membranes

Anion transport in erythrocytes was inhibited using the highly specific inhibitor DIDS that binds covalently to the band 3 protein (anion channel) of membranes [34]. Cells were incubated at 23 °C in NaCl-saline, containing 10 mM borate buffer, pH 8.5 and 10 µM DIDS, hematocrit 0.03, at dark, for 15 min. Prior to use, the treated cells were washed thrice in excess NaCl-saline and inhibition verified as follows. Addition of erythrocytes to an isotonic 20 mM NaCl sucrose medium is known to result in a rapid acidification of the outer medium due to the exchange diffusion of inner Cl^- for outer OH^- through the anion channel of the membranes [35]. Compared to intact cells, the addition of DIDS-treated cells to such a medium resulted in a reduced acidification rate and amplitude which was used to assess the inhibition of the anion channel obtained.

References

1 Comis, L.; Carter, S.: Cancer Treat. Rev. **1**, 285 (1974)
2 Michael, B.; Atkins, M.: Current Opinion in Oncology **9**, 205 (1997)
3 Vorobiov, A.; G. Falkson, G.: Cancer Ther. Update **11**, 3 (1982)
4 Hopwood, J.; Stock, A.: Chem. Biol. Interact. **4**, 31 (1971)
5 Pyrhonen, S.; Hahka-Kemppinen, M.; Muhonen, T.: J. Clin Oncol. **10**, 1919 (1992)
6 Kann, H., in: Prestayko, A. W.; Grooke, S. T.; Baker, L. H.; Carter, S. K.; Shein, P. S. (Eds.): Nitrosoureas: current status and new developments, Academic Press, New York 1981
7 Kolar, G.: in Schmahl, D.; Kaldor, J. M. (Eds): Carcinogenicity of alkylating cytostatic drugs **78**, p. 111 IARC, Lyon 1986
8 Stahl, W.; Denkel, E.; Eisenbrand, G.: Mutat. Res. **206**, 459 (1988)
9 Mitchell, J.; Biaglow, J.; Russo, A.: Pharmac. Ther. **39**, 274 (1988)
10 Giaccia, A.; Lewis, A.; Denko, N.; Cholon, A.; Evans, J.; Walden, C.; Stamato, Th.; Brown, J.: Cancer Res. **51**, 4463 (1991)
11 Thulin, H.; Zorcec, V.; Segerback, D.; Sundwall, A.; Tornquist, M.: Chem. Biol. Interact. **99**, 263 (1996)
12 Black, R.; Harrison, J.; Read, R.: Xenobiotica **27**, 11 (1997)
13 Lassmann, G.; Herrmann, A.; Raikov, Z.; Muller, P.: Cancer Biochem. Biophys. **9**, 169 (1987)
14 Friedman, S.; Skehan, P.: Cell membranes: Targets for selective antitumor chemotherapy. Novel Approaches to Cancer Chemotherapy, Academic Press, New York 1984
15 Arancia, G.; Donelli, G.: Pharmacol. Res. **24**, 205 (1991)
16 Gadgeva, V.; Raikova, E.; Raikov, Z.; Vlaikova, T.: Eur. J. Cancer. **27**, S55 (1991)
17 Li, Z.; Brendel, M.: Mutat. Res. **315**, 139 (1994)
18 Gray, P.; Lewis, K.; Masta, A.; Phillips, D.: Biochem. Pharmacol. **47**, 581 (1994)
19 Brake, W.; Pappas, B.: Brain Res. Dev. Brain Res. **18**, 289 (1994)
20 Joyner, S.; Kirk, K.: Am. J. Physiol. **267**, R773 (1994)
21 Ivanov, I.: Biochim. Biophys. Acta, **1415**, 349 (1999)
22 Byrne, M.; Broomfield, C.; Stites, W.: J. Protein Chem. **15**, 131 (1996)
23 Sosnovsky, G.; Shu, W.; Rao, N.: Z. Naturforsch. **42c**, 921 (1987)
24 Raikov, Z.; Gadzheva, V.; Koch, M.; Kolar, G.: OPPI **25**, 473 (1993)
25 Gadjeva, V.; Raikova, E.; Raikov, Z.; Ivanova, Ts.; Stefanova, M.: Com. Rend. Acad. Bulg. **42**, 131 (1989)
26 Bodemann, H.; Passow, H.: J. Membr. Biol. **8**, 1 (1972)
27 Savitz, D.; Siedel, V.; Solomon, A.: J. Gen. Physiol. **48**, 48 (1964)
28 Ivanov, I.; Benov, L.: J. Therm. Biol. **17**, 381 (1992)
29 Ivanov, I.: Membr. Cell Biol. **11**, 45 (1997)
30 Ivanov, I.: Gen. Phys. Biophys. **18**, 165 (1999)
31 Brandts, J.; Erickson, L.; Lysko, K.; Schwartz, A.; Taverna, R.: Biochemistry, **16**, 3450 (1977)
32 Ilani, A.; Granoth, R.: Biochim. Biophys. Acta **1027**, 199 (1990)
33 Yang, X.; Kamino, K.: Jpn. J. Physiol. **45**, 723 (1995)
34 Cabantchik, J.; Rothstein, A.: J. Membr. Biol. **15**, 207 (1974)
35 Macey, R.; Adorante, J.; Orme, F.: Biochim. Biophys. Acta **512**, 284 (1978)

Received December 12, 1999
Accepted January 5, 2000

Ivan T. Ivanov
Dept. Physics and Biophysics
Medical Institute
Thracian University
Armeiska Str. 11
6000 Stara Zagora
Bulgaria
ivanov_it@hotmail.com