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SHORT COMMUNICATIONS

Transport mechanism of DIAM 3, a new anticancer drug, in human glioblastoma (U 251) cells in culture

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It has been recently emphasized that polyamine analogues might offer interest in cancer chemotherapy by interfering with polyamine transport [1, 2]. Moreover, we have recently demonstrated that DIAM 3, a polyamine vectorized cyclophosphazene, exerted dose dependent antitumoral effects in mice transplanted with P 388, L 1210 and P 815 cells [3] and in nude mice xenografted with human malignant glioblastoma cells [4]. Therefore, this study was undertaken to elucidate the transport mechanism of this drug in cultured human glioblastoma (U 251) cells.

Materials and Methods

The drug, patented as DIAM 3 [5], was prepared by Pierre Fabre Medicament Co. (Castres, France) as the licensee. The molecular structure of DIAM 3 displays an Aziridinyl (Az) bearing cyclophosphazenic moiety linked to 1,3-diaminopropane in a SPIRO configuration, i.e. $N_3P_3Az_4$ [HN-$(CH_2)_3$-NH] (Fig. 1). The details of synthesis and chemical characterization including NMR and X-ray studies of this drug have been published elsewhere [3, 6]. DIAM 3 in the form of [^{14}C]DIAM(^{14}C-isotope tagged in Az group; sp. act. 4.9 mCi/mmol) was supplied as dry powder by Isotop Chim. (Gonagobie, France). Before using, the drug was reconstituted with phosphate buffered saline (pH 7.4). The glioblastoma (U 251) cells are maintained in our laboratory in RPMI 1640 medium containing 10% foetal calf serum. When the cells were near to confluence, they were used for experimental manipulations. The cells were incubated in serum free medium for 15 min containing different test reagents. The incubations were terminated by washing the cells with PBS-D (phosphate buffered saline, pH 7.4 containing non-radioactive DIAM 3; 1 mM) extensively in order to eliminate [^{14}C]DIAM 3 bound to the exterior of the plasma membranes. The cells were detached by adding trypsin-EDTA (0.5%) and further washed three times with PBS (phosphate buffered saline, pH 7.4) by centrifuging at 2000 *g* for 15 min. No radioactivity was lost during cell detachment from the substratum. The radioactivity retained by the cells was measured by dissolving them in Soluene 100 (1.0 mL). Protein concentrations were determined according to Lowry *et al.* [7].

Results and Discussion

We have recently characterized the mechanisms of polyamine transport in human red blood cells [8] and in other cells including U 251 cells [9]. The polyamine transport was observed to be Na^+ dependent and since DIAM 3 is a polyamine-vectorized drug, we were tempted to assess the sodium dependency of DIAM 3 transport. Figure 2 shows that the mechanism of DIAM 3 transport is sodium dependent. The uptake of [^{14}C]DIAM 3 is reduced by decreasing extracellular sodium concentrations. At 0% of extracellular sodium concentration added, there is, however, 20% transport of DIAM 3 and this may be due to the continuous presence of Na^+ from sodium bicarbonate in the culture medium. Sodium has been shown to be the source of electrochemical gradient energy required for the transport of amino acids, D-glucose and polyamines [8, 10, 11]. Na^+ electrochemical gradient is involved, particularly in the transport of System A and System ASC amino acids [10]. We also assessed the possibility of DIAM 3 transport to be operated via System A or System ASC. We used α-amino isobutyric acid (AIB), a non-metabolizable substrate for System A transport. The cells were preloaded either with AIB or asparagine (a candidate for System ASC) or with lysine (Ly^+ System) and it was observed that prior incubation with either of the amino acids did not affect the DIAM 3 transport appreciably (Table 1). These observations suggest that DIAM 3 transport is not shared by System A, System ASC or System Ly^+ amino acids. In U 251 cells, the polyamine transport is also Na^+ dependent which is not influenced by prior incubations with none of the amino acids used in the present study [9]. If the Na^+ electrochemical gradient is involved in DIAM 3 transport, the dissipation of this gradient with specific antibiotics

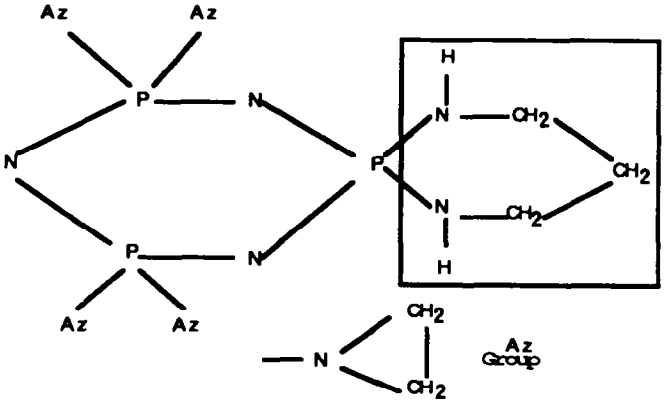

Fig. 1. The molecular pattern of DIAM 3. The drug displays four aziridinyl (Az) groups and one 1,3-diaminopropane moiety (inset) grasped on phosphorous atoms of a 6-membered phosphagenic ring.

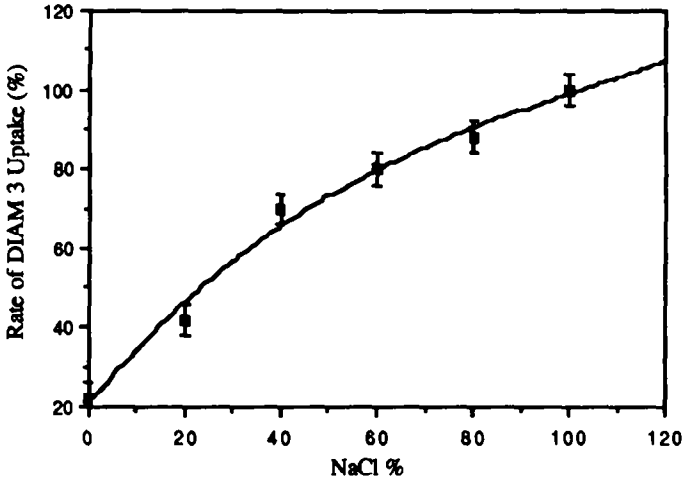

Fig. 2. Sodium dependency of DIAM 3 transport in human glioblastoma (U 251) cells. The cells (2×10^6) were incubated for 15 min in serum free RPMI 1640 medium in which NaCl was replaced iso-osmotically with choline chloride (from 0 to 100%). Hence 100% of Na^+ concentration corresponded to 111 mM of NaCl added in the culture medium [6]. After terminating the reaction, the cells were washed and the radioactivity was counted as described in Materials and methods. Data are means ± SD of triplicate assays.

should inhibit the uptake process. In Table 1 two classes of ionophores, mobile ion carriers and channels formers, with different cation specificity are used to probe this notion. Valinomycin and A 23187 possess cationic specificities for K^+ and Ca^{2+}, respectively, while gramicidin possesses specificity for K^+ and Na^+. Table 1 shows that gramicidin and Ca^{2+} ionophores inhibit the DIAM 3 transport while valinomycin stimulates slightly and this suggests that a membrane potential may be involved in the transport of DIAM 3. These observations are consistent with another study in which aziridinocyclophosphazenes reduced the membrane potential as well as membrane potential-dependent amino acid transport in *Streptococcus pneumoniae* [12]. Several studies have demonstrated that Na^+ dependent transport of carbohydrates [11], amino acids [10] and polyamines [9] possess functional-SH groups which are sensitive to sulfhydryl reagents. This observation together with Na^+ dependency of DIAM 3 transport led us to investigate the effects of sulfhydryl reagents on the DIAM 3 transport (Table 2). Prior incubation with NEM and pCMBS (oxidized sulfhydryl reagents) is followed with a lowering (54 and 47% respectively) of DIAM 3 transport while a further treatment of NEM and pCMBS treated cells with DTT (reduced sulfhydryl reagent) is followed with a restoration of DIAM 3 transport (76 and 82%, respectively) towards normal values. These observations suggest that functional-SH groups are involved in DIAM 3 transport.

The properties of DIAM 3 transport, Na^+ dependency, independency on System A and System ASC or on other amino acids, sensitiveness to sulfhydryl reagents and involvement of membrane potential, resemble with properties of polyamine transport in U 251 cells [9] and these observations further led us to speculate that, perhaps, DIAM 3 is utilizing the polyamine transport system present in these cells. Therefore, we further investigated the effects

Table 1. Effects of System A and other amino acids and ionophores on DIAM 3 transport in human glioblastoma (U 251) cells

Addition	Uptake of DIAM 3 (pmol/mg protein)	Per cent transport
None (control)	123.6 ± 4.7	100
AIB	113.0 ± 4.3	91
Asp	116.9 ± 4.4	93
Ly	120.4 ± 5.5	97
Valinomycin (10 μM)	137.5 ± 1.5	111*
A 23187 (1 μM)	102.9 ± 7.4	82*
Gramicidin (10 nM)	100.1 ± 3.0	80*

The cells (2×10^6) in culture were added with serum free RPMI 1640 medium containing amino acids i.e. AIB (α-aminoisobutyric acid), Ly (lysine) or Asp (asparagine) each at 1 mM concentration for 2 hr. The cells were added with medium containing [^{14}C]DIAM 3 (100 nM) and incubated for 15 min. The transport assays were performed as described in Materials and methods. Data are means ± SD of triplicate assays.

* These values differ significantly in comparison with the control according to the Mann-Whitney test of significance ($P < 0.01$).

Table 2. Effects of sulfhydryl reagents on DIAM 3 transport in human glioblastoma (U 251) cells

Addition	Uptake of DIAM 3 (pmol/mg protein)	Per cent transport
None (control)	364.9 ± 59.8	100
NEM	197.6 ± 49.0	54*
pCMBS	174.0 ± 53.7	47*
NEM + DTT	280.4 ± 40.3	76
pCMBS + DTT	301.5 ± 47.5	82

The cells (4×10^6) in culture were preincubated with sulfhydryl reagents i.e. NEM (*N*-ethylmaleimide; 5 mM) or pCMBS (*p*-chloromercuribenzenesulfonate; 50 mM) for 20 min in RPMI 1640 medium prior to the addition [^{14}C]DIAM 3 (100 nM) with the exception in the last two groups where cells treated with NEM or pCMBS were further incubated with DTT (dithiothreitol; 10 mM). The transport assays were performed as described in Materials and methods. Data are means ± SD of triplicate assays.

* These values differ significantly in comparison with the control according to the Mann-Whitney test of significance ($P < 0.01$).

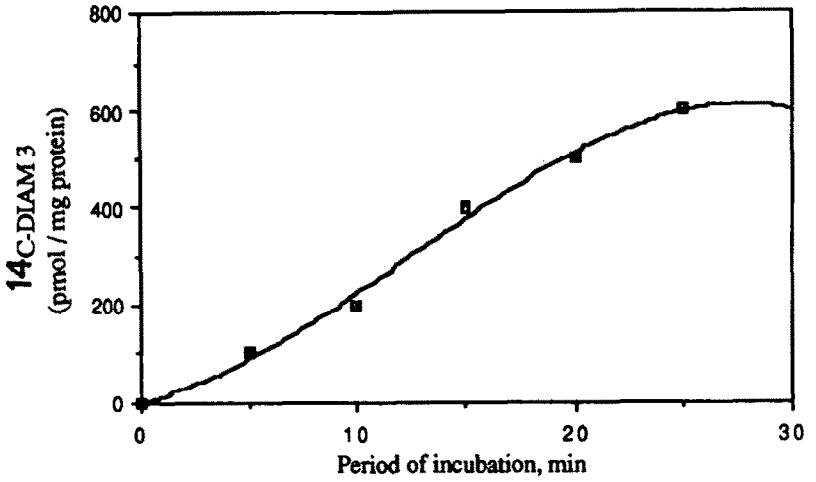

Fig. 3. Time course of DIAM 3 uptake by U 251 cells. The cells (2×10^6) were incubated with [^{14}C]DIAM 3 (100 nM) for different times ranging from 0 to 25 min. The assays were terminated by washing the cells with PBS-D (phosphate buffered saline, pH 7.4 containing non-radioactive DIAM 3, 1 mM). Later on, the cells were detached from the substratum and radioactivity internalized into the cells was measured as described in Materials and methods. Data are means of duplicate samples. The graph was set by the computer.

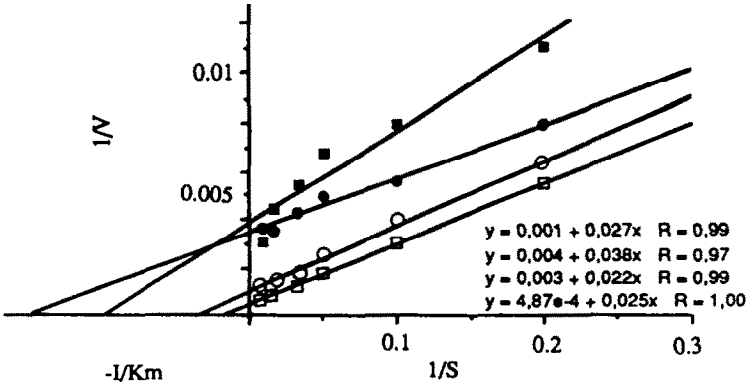

Fig. 4. Apparent kinetics of [^{14}C]DIAM 3 in human glioblastoma (U 251) cells. The cells (2×10^6) were added with serum free RPMI 1640 medium containing increasing concentrations of [^{14}C]DIAM 3 (from 0 to 100 $\mu M \times 10^{-3}$) with or without (□) fixed concentrations (10 mM each) of Put (○), Spd (●) or Spm (■). After 15 min the reaction was terminated and radioactivity internalized was measured as described in Materials and methods. The abcissa and ordinate of the graph represent $1/S$ ($\mu M \times 10^{-3}$) and $1/V$ (pmol/mg protein/15 min), respectively. Data are means of triplicate assays.

of competitions of polyamines with [^{14}C]DIAM 3 transport in U 251 cells. The uptake of [^{14}C]DIAM 3 by U 251 cells was linear till 25 min of incubation (Fig. 3). The studies on the kinetics of [^{14}C]DIAM 3 demonstrate that polyamines at 10 mM concentrations reduced the K_m of DIAM 3 uptake, shifting K_m from 25 $\mu M \times 10^{-3}$ (control) to 22, 10 and 15 $\mu M \times 10^{-3}$ in the presence of Put, Spd and Spm, respectively (Table 3 and Fig. 4). These observations on kinetics indicate that polyamines increase the affinity of DIAM 3 for U 251 cells. The inhibition of K_m by polyamines in this study appears to be of non-competitive type because both V_{max} and K_m are decreased. In a similar study, Porter *et al.*

Table 3. The effects of polyamines on apparent kinetics of DIAM 3 in human glioblastoma (U 251) cells

Addition	K_m ($\mu M \times 10^{-3}$)	V_{max} (pmol/mg protein/15 min)
None (control)	25	650
Put	22	400
Spd	10	150
Spm	15	140

For legends, see Fig. 3. Data are derived from Fig. 3.

[13] observed a competitive type of inhibition of spermidine uptake by L 1210 cells with a series of diamine homologues of putrescine and triamine homologues of spermidine and it was suggested that these polyamine homologues were interfering with polyamine transport. They further observed that one of the polyamine analogues could inhibit the cell proliferation with $IC_{50} < 10$ mM [14]. Though in the present study polyamines do not show a competitive type of inhibition of DIAM 3 uptake by U 251 cells, nevertheless, by considering the results on the similarities between polyamine [9] and DIAM 3 uptake by these cells, we may speculate that DIAM 3 would be utilizing the polyamine transporter protein to enter the cells.

We do not know about the metabolism of DIAM 3 by U 251 cells since the studies on this drug are limited. In another experiment, after 10% trichloroacetic acid precipitation of U 251 cells containing [^{14}C]DIAM 3, we observed no radioactivity in the acid soluble material and this indicates towards the covalent binding of DIAM 3 with cell proteins. As regards the efflux of the drug, we observed that after transfering the [^{14}C]DIAM 3 loaded cells into a drug free RPMI 1640 medium, no radioactivity was lost during the chase of every 15 min till 2 hr.

Further studies are needed on the localization and catabolism of this new anticancer drug by U 251 or other mammalian cells.

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Effect of 5-aminosalicylic acid on ferrous sulfate-mediated damage to deoxyribose

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Ulcerative colitis is a recurrent inflammation of the colon and rectum characterized by diffuse ulcerations, crypt abscesses, decreased mucous production, subepithelial hemorrhage, and infiltration of large numbers of phagocytic leukocytes (monocytes, macrophages, and granulocytes). Oral administration of sulfasalazine (SAZ) has been proven to be effective in attenuating the mucosal injury associated with this inflammatory disease. Sulfasalazine passes unmodified through the upper gastrointestinal tract into the colon where it is metabolized by enteric bacteria to yield 5-aminosalicylic acid (5-ASA) and sulfapyridine (SP). Although it is now well accepted that 5-ASA is the pharmacologically active moiety of SAZ [1], the mechanisms by which 5-ASA exerts its beneficial effect remain speculative.