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Attempts at the Production of More Selective Antitumourals. Part III*. Aziridinocyclophosphazenes Linked to the Polyamines 1,5-Diaminopentane (Cadaverine) and Higher Cousins

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Abstract

In an attempt to design antitumour cyclophosphazenes of improved specificity by linking them to biogenic polyamines as tumour finders, we studied the binding of $N_3P_3Az_5Cl$ to cadaverine and its cousins. Synthesis, NMR and mass spectrometry of the bino vectorized drugs (in which two $N_3P_3Az_5$ active principles are linked to the diamine in a bino configuration) are described. Unfortunately, these vectorized drugs display such poor water solubility (less than 4 g l^{-1}) that studies of their biological activities could not be performed. In other words, vectorization of aziridinocyclotriphosphazenes through natural polyamines as tumour finders in a bino configuration is not suitable for industrial purposes, in contrast with what was previously demonstrated when vectorization occurs in spiro and dispiro-bino configuration.

Introduction

Any allopathic remedy, even when prescribed carefully, always induces side-effects in treated patients. Drugs are indeed extraneous bodies for the system. Thus, in allopathy, even with sub-acute, chronic and limited schedules, there is actually a dramatic balance between therapeutic benefits and penalizing side-disorders.

This is especially true in cancer chemotherapy, since antitumour agents usually have low therapeutic indexes. In other words, anticancer drugs must normally be used close to their toxic doses, thus inducing huge side-effects. In that case, such side-effects are a consequence of the poor selectivity of drugs for the malignant cells, a too large amount of the injected dose being spread out over the rest of the body, *i.e.* over the healthy cells. This poor selectivity constitutes a challenge to both the chemists who are in charge of designing new drugs and the clinicians who are daily in charge of prescribing them. Many multidisciplinary efforts have been made during the last few years to enhance the selectivity of anticancer drugs, essentially through covalent binding either to monoclonal antibodies (immuno-globulins) [1] or to natural polyamines (mainly putrescine, cadaverine, spermidine and spermine) [2], antibodies and polyamines playing the role of tumour finders and, in few cases, of homing heads. These two new approaches are actually diversely successful but they are some of the newest ways to escape from the uncomfortable dilemmas found in allopathy in cancer chemotherapy nowadays.

Anticancer inorganic ring systems [3] do not escape the problem and several attempts at the production of more selective agents have been achieved in our laboratory recently, polyamines being selected as tumour finders. Interest in such attempts is enforced by the fact that these inorganic ring systems, initially designed as anticancer agents, also exhibit promising immuno-modulating properties which may help the treatment of auto-immune diseases like disseminated erythematous lupus [4], glomerulo-nephritis [5] and blood-sugar [6]. The slowly developing character of such diseases requires a chronic regimen for quite long periods; the dose to be injected daily has to be as far as possible from the maximal tolerated one and, consequently, only drugs with very high therapeutic index (that is very selective for malignant cells) have any chance of being developed for clinical uses.

In an attempt to increase the selectivity of cyclophosphazenes and decrease their toxicity for normal tissues and thus obtain compounds possessing a high therapeutic index, we explored as a first step the linkage to 1,3-diaminopropane [7–9] and 1,4-diaminobutane (putrescine) [9, 10]. Covalent binding of the polyamine to the drug occurs in both cases quite neatly, leading to unique vectorized drugs in which diamines are in a spiro configuration. The benefits of such a targeting through polyamines in a spiro configuration were demonstrated by the

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EORTC Screening and Pharmacology Group for anti-tumour activity [9, 11] and by clinicians of the INSERM 269 Unit (Purpan Hospital, Toulouse, France) for immuno-modulating effectiveness [12]: a given therapeutic effect needs 15 times less of the drug when vectorized, with a concomitant huge reduction of side-effects, mainly thrombocytopenia, owing to their dose-dependent character.

As a second step in exploring the potentiality of polyamines as tumour finders, we linked 'nude' anti-cancer immuno-modulating cyclophosphazenes to spermine. Final super-molecules were once more obtained quite neatly, spermine being covalently bound to two cyclophosphazenic active principles in a dispiro-bino configuration [13]. Incidentally, spermidine also links two active principles in a spiro-bino configuration [14]. The benefits of such a targeting through spermine were studied both by the EORTC Group and by the INSERM 269 Unit: they are less than in the previous case, *i.e.* with polyamines in a spiro configuration, for antitumour properties (active doses for a given therapeutic effect being only five times less for vectorized drugs) but higher than in the previous case for immuno-modulating properties, effective doses being divided by sixty [15].

In order to attempt further improvements on anti-tumour and immuno-modulating properties of cyclo-phosphazenes through polyamine vectorization, we subsequently investigated covalent linkages of these inorganic ring systems to cadaverine and higher cousins. This contribution reports on the synthesis and physico-chemical identity of super-molecules obtained in this way.

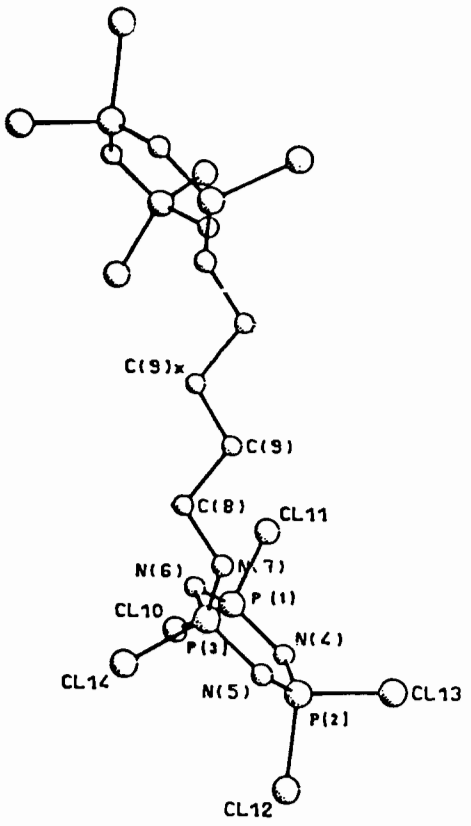


Fig. 1. A perspective view of bino-4 with numbering of atoms (half molecule).

Experimental

Synthesis of Chlorinated Precursors

The synthesis of super-molecules vectorized by cadaverine and higher cousins goes through the following two-step route:

(1) reaction of polyamines with hexachlorocyclo-triphosphazene, $N_3P_3Cl_6$, under (1:2) stoichiometric conditions;

(2) peraziridinylation of these chlorinated pre-cursors.

Reactions of $N_3P_3Cl_6$ with cadaverine, $H_2N-(CH_2)_5-NH_2$, and higher cousins, $H_2N-(CH_2)_n-NH_2$, with $n > 5$, under (2:1) conditions lead to unique final products in which two $N_3P_3Cl_5$ moieties are bridged through a $[HN-(CH_2)_n-NH]$ entity in a two-ring assembly structure [16]. This new type of configuration for the diamino-ligand, called bino, was revealed by concerted use of mass spectrometry and ^{31}P, ^{13}C and ^{1}H high resolution NMR [17]. X-ray structure of bino-4, *i.e.* $N_3P_3Cl_5[HN-(CH_2)_4-NH]-Cl_5P_3N_3$, was achieved [18] and is shown in Figs. 1 (molecule) and 2 (unit cell).

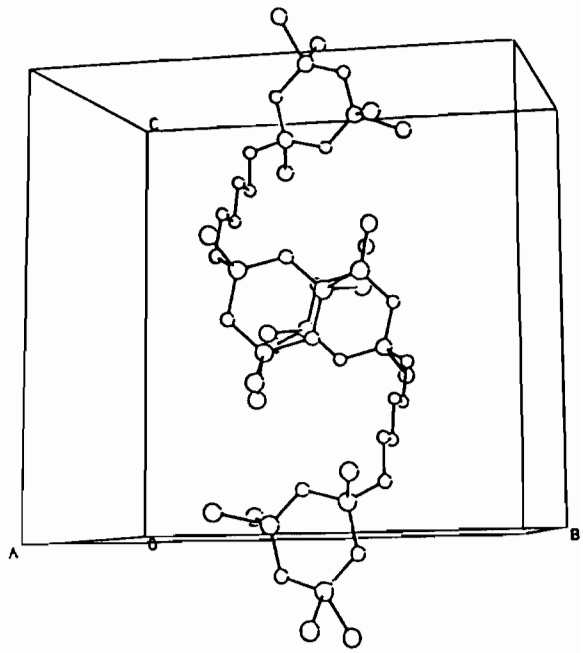


Fig. 2. A perspective view of the unit cell of bino-4 along the C9–C9* direction.

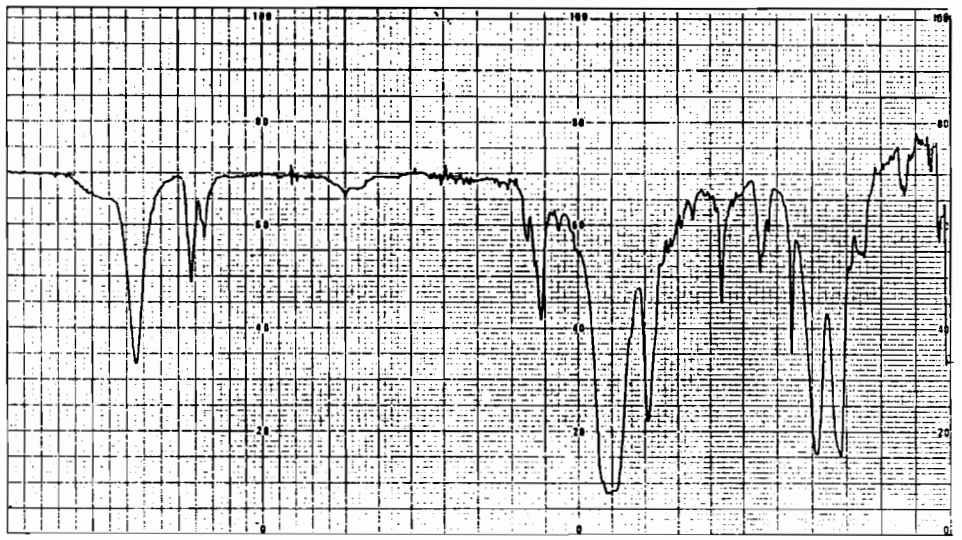
These bino-chlorinated precursors are obtained once more in a quite neat way, under the express condition of (2:1) stoichiometric conditions in anhydrous ethyl ether as the solvent and in the presence of an amount of triethylamine just enough to remove hydrogen chloride. A suitable excess of diamine leads indeed to complex mixtures in which ^{31}P high resolution NMR reveals dibino, tribino and higher polybino moieties [19]. These bino two-ring assembly structures may be considered as the first step to cyclophosphazene polymerization (cross-linking), according to Allcock [20].

Synthesis of Super-molecules Vectorized through Cadaverine and Higher Cousins

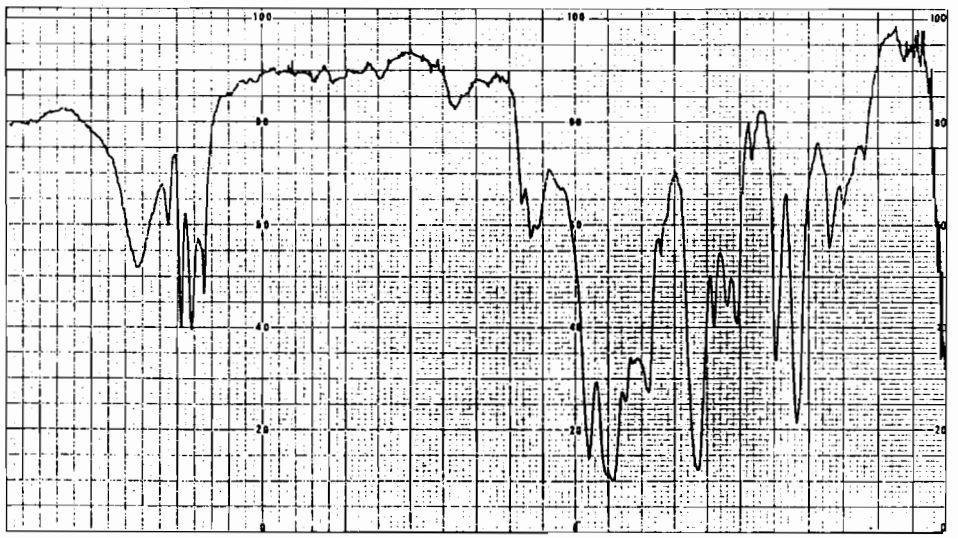
These super-molecules are obtained on peraziridinylation of the bino-chlorinated precursors just described.

The nomenclature is as follows: a bino-chlorinated precursor will be coded as bino-*n*-Cl, the corresponding aziridinylated drug being coded as bino-*n*-Az.

Let us now describe the synthesis of bino-8-Az. 13.5 mmol of aziridine (*i.e.* 100% excess with respect to the quantity needed both for persubstitution and removal of hydrogen chloride) in 50 ml of anhydrous



(a) BINO 8 Cl



(b) BINO 8 Az

Fig. 3. IR spectra (Perkin-Elmer 683) of (a) bino-8-Cl and of (b) bino-8-Az.

Et_2O are added dropwise in 2 h to 4.5 mmol (3.45 g) of bino-8-Cl in 200 ml of the same solvent. The medium is stirred under argon pressure in an ice-bath. The reaction takes 4 days and is considered complete when the IR frequencies (525 and 595 cm^{-1}) of the P–Cl bonds in bino-8-Cl (Fig. 3) have disappeared. The hydrochloride is then filtered off, solvent is removed *in vacuo* at 25 °C to give a colourless syrupy residue. The oily character of the final crude product is due to clathration by Et_2O. First, the residue is washed with 20 ml of cold water to remove traces of hydrochloride. Extraction with 150 ml of Et_2O three times and removal of the solvent *in vacuo* give a hydrochloride-free colourless oil which is stirred for 2 days with 300 ml of n-heptane, in order to remove Et_2O from the clathrated moiety. Bino-8-Az is so obtained as a white powder after removal of the solvent, 1.2 g (34%), m.p. 85 °C. The analytical data are highly consistent with the bino-8-Az structure, *i.e.* $N_{18}P_6C_{26}H_{54}$.

The ^{31}P NMR spectrum, recorded on a Bruker WH 90 instrument, exhibits, as expected, a doublet at 38.52 and 37.55 ppm (PAz_2 entities) and a triplet at 30.69, 29.72 and 28.76 ppm (PAzNH moieties) in $CDCl_3$ with H_3PO_4 85% as a standard. The coupling constant $^2J_{PP}$ is equal to 35.3 Hz.

Such a pure A_2B NMR spectrum is definite proof of the achievement of peraziridinylation. Indeed, Fig. 4 shows ^{31}P NMR spectra on days 2 and 4 of the synthesis of bino-8-Az: reaction is achieved on day 4 but not on day 2; the spectrum reveals some ABC-type sub-structures with triplets around 20.24 ppm (PCl_2 units), 42.44 ppm (PAzCl units), together with the corresponding PAzNH and PAz_2 triplets beneath the genuine doublet-triplet A_2B pattern of the pure bino-8-Az. Thus, it is an easy matter to check the spectrum for the presence of any possible non-persubstituted contaminant.

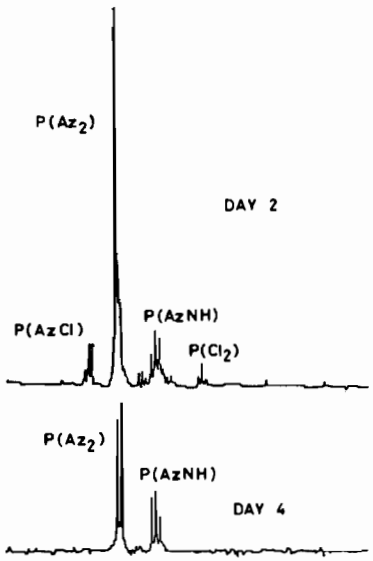


Fig. 4. ^{31}P NMR spectra on days 2 and 4 of the peraziridinylation of bino-8-Cl leading to bino-8-Az.

The synthesis of bino-*n*-Az super-molecules has to be performed in anhydrous Et_2O as the solvent and with an excess of aziridine to remove hydrogen chloride. Indeed, reactions in other solvents or mixtures of solvents lead either to smaller yields or to decomposition of the final product: for example, aziridinylation in THF breaks the diamino bridge of bino-*n*-Cl, leading to pure hexaziridinocyclotriphosphazene, $N_3P_3Az_6$ (MYKO 63) [3]. The same reaction using a (9:1) mixture of n-hexane and CH_2Cl_2 (chosen in order to avoid clathration), occurs very slowly and leads after one week to complex mixtures of partly substituted moieties. On the other hand, the use of other agents for removal of HCl is less convenient than aziridine itself: Et_3N gives a hydrochloride which cannot be fully removed from the final crude product, even through SiO_2 column chromatography. Ammonia cannot be used because it reacts in part with the bino-*n*-Cl starting material to give some fused amino-aziridino substituted materials, as demonstrated by ^{31}P NMR. Figure 5 shows the ^{31}P

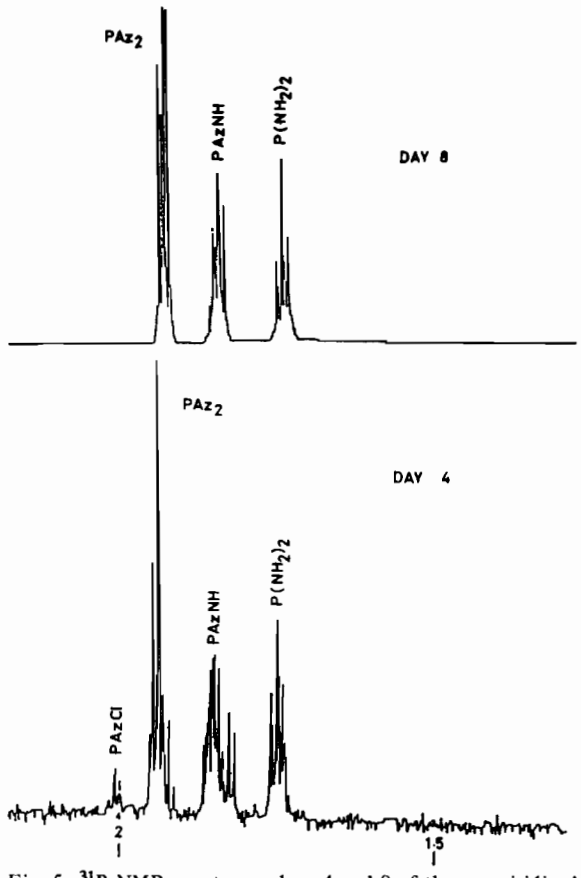
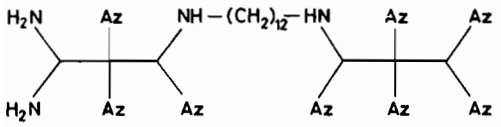


Fig. 5. ^{31}P NMR spectra on days 4 and 8 of the peraziridinylation of bino-12-Cl in the presence of ammonia for removal of HCl.

NMR spectrum (on day 4) of the final product obtained upon peraziridinylation of bino-12-Cl in the presence of NH_3: the multiplet at around 18.5 ppm ($P(NH_2)_2$ species) proves that ammonia reacts first on bino-12-Cl leading to the gem-diamino (or symmetrical tetramino) derivative (as is the case when bubbling NH_3 through Et_2O solutions of $N_3P_3Cl_6$); the aziridine reacts only in sequence to substitute the remaining chlorine atoms. Furthermore, some PAzCl moieties are revealed on day 4 which disappear only on day 8, the final spectrum (Fig. 5, day 8) at 36.43 MHz displaying three multiplets at around 18.5 ($P(NH_2)_2$), 29 (PAzNH) and 38 (PAz_2) ppm which are actually, when recorded at 101.27 MHz, superimposition of the genuine A_2B doublet-triplet spectrum for bino-12-Az and of the ABC-type (three doublets of doublets) spectrum for the gem-diamino entity:



Electron-impact mass spectra of bino-*n*-Az derivatives could not be recorded in the usual way, as the molecular weights were larger than 700. We previously demonstrated that the EI technique works under saturated conditions for amplifier and multiplier, that is under questionable conditions, for revealing molecular M^+ peaks. Thus, DCI mass spectrometry was used, which has been recently developed by our group for assessing molecular structures and monitoring purity in cyclophosphazenic series [21, 22].

DCI mass spectrum of bino-8-Az is reported in Fig. 6. The molecular ion MH^+ is observed at m/z 833 (with its M, $2NH_4^+$ m/z 869 (2.3%) satellite). Incidentally, this MH^+ peak is the base peak (I = 100%).

The main fragmentation route proceeds through the loss of $N_3P_3Az_5$, leading to the fragment m/z 487. The successive loss of 1NH, 1 to 8 CH_2 and 1NH again occurs step-by-step giving maximal peaks at m/z 472 (33.8%), 458 (49.4%), 444 (30.3%), 430 (22.7%), 416 (27.8%), 402 (14.3%), 388 (42.5%), 374 (29.1%), 362 (21.7%) and 345 (15.5%). The loss of 1 to 3 Az groups (m/z 42) from m/z 345 gives peaks at 302 (33.2%), 261 (4.5%) and 219 (5.7%).

A second fragmentation route occurs through the loss of 1 to 8 Az groups from the MH^+ peak, giving maximal peaks at m/z 790 (5.0%), 747 (44.9%), 704 (10.1%), 661 (56.5%), 620 (19.1%), 576 (10.4%), 535 (4.3%) and 483 (8.6%).

Thus, it is a simple matter to check for the absence of any other derivative owing to the simplicity of the spectrum as a whole.

Biological Activities

The bino-*n*-Az (n = 6 to 12) super-molecules reported here are poorly water-soluble (about 4 g l^{-1}), even when freshly prepared. They cannot be easily dissolved in sterile saline for i.p. and/or i.v. administrations.

Immuno-modulating properties of these super-molecules were tested at the INSERM 269 Unit (Purpan Hospital, Toulouse, France) on murine glomerulo-nephritis induced by chronic injections of lipopolysaccharide (LPS) [5].

The main results which could be expected from these experiments were the following: (i) Does bino-*n*-Az modulate or not the polyclonal activation of lymphocytes B and prevent glomerulo-nephritis, as do

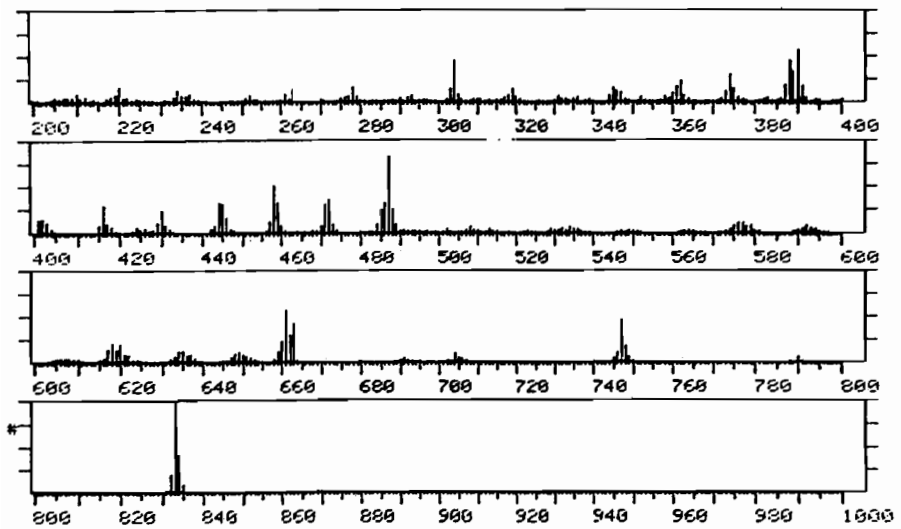


Fig. 6. 70 eV DCI mass spectrum of bino-8-Az.

other cyclophosphazenes, vectorized or not ?; (ii) is this effectiveness, if any, dependent on the length of the methylene chain of the bridge and/or on the 'odd or even' n value? The influence of the length of the bridge was actually suggested to us by the fact that the prior target of biologically active cyclophosphazenes lies on the cell membrane [23] and not on the DNA [24]: thus, lengthening of the diamino bridge, inducing a higher lipophilic character for the drug, would be a favourable argument for higher activity.

Actually, the poor solubility in water and/or in saline (see above), probably due to the lipophilic character of the methylene chain into the bino bridge, is a factor which drastically limits the potential use of such vectorized drugs for further industrial development. The largest dose which can be inoculated in mice being about 0.2 ml per mouse, doses larger than 200 mg/kg cannot be injected in mice for *in vivo* tests. Unfortunately, the highest non-lethal doses (LD_0) of such bino-Az drugs are so high (larger than 3000 mg/kg in KLUCEL JF, *i.e.* hydroxypropylcellulose from Hercules Co. as the 'solvent' [25]) that neither antitumoural activities nor immuno-modulating effectiveness could be revealed by the i.p. route.

Actually, this is a failure. Vectorization through cadaverine and higher cousins in a bino configuration leads to super-molecules which are so weakly toxic and so poorly soluble in saline that their development for eventual use in clinics is hopeless.

Thus, vectorization of anticancer aziridinocyclotriphosphazenes through biogenic polyamines both in a spiro (DIAMS super-drugs) and in a dispiro-bino configuration (SPM super-drugs) is a success, but vectorization through cadaverine and higher cousins (BINOS super-drugs) is a failure. Further improvements within the vectorization of aziridinocyclotriphosphazenes through suitable other functionalized diamines in a bino configuration, which would lead to super-drugs more soluble in saline, are now in progress, according to Montanari [26].

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