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Antitumour Activity of Some Cyclophosphazenes

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Abstract—*Among six cyclophosphazenes tested for antitumour activity against mouse P388, L1210 and B16 tumours respectively, activity was shown by the hexaziridinocyclotriphosphazene* ($N_3P_3Az_6$; *all three tumours*), *the octaziridinocyclotetraphosphazene* ($N_4P_4Az_8$; *all three tumours*) *and the octapyrrolidinocyclotetraphosphazene* ($N_4P_4Pyrro_8$; *P388 tumour*). *The most effective in each case is the hexaziridino trimer compound,* $N_3P_3Az_6$, *which has the advantage of high water solubility and activity both by the i.v. and by the i.p. route. Three compounds,* $N_3P_3Cl_6$, $N_4P_4Cl_8$ *and* $N_3P_3Pyrro_6$ *were inactive. The mode of action of these drugs on DNA and a possible structure–activity relationship are briefly discussed.*

INTRODUCTION

DESPITE the fact that Cernov *et al.* [1] reported in 1959 on the "positive activity" of some cyclophosphazenes against M1 and S180 sarcomas, no further developments were performed so far in this field.

We had previously reported on the electronic structure [2–4] and the physicochemical behaviour [5–7] of compounds containing such phosphorus–nitrogen rings and now report on their antitumour activities in relation to these fundamental studies.

In considering the geometrical structure of the hexachlorocyclotriphosphazene, $N_3P_3Cl_6$ (Fig. 1), it may be noted that such a cyclophosphazene is characterised by two specific

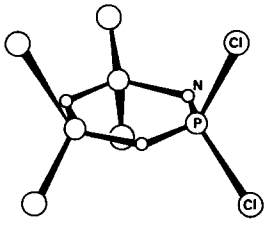

Fig. 1. Geometrical structure of $N_3P_3Cl_6$.

structural features relevant to potential antitumour activity: (i) each phosphorus carries two chlorine atoms the distance between which is 3.1 Å [8], close to the 3.4 Å one in Rosenberg's PDD, *cis*-$(NH_3)_2PtCl_2$ [9]; thus, bifunctional alkylation of DNA could be expected if the lability of P–Cl and Pt–Cl bonds with respect to hydrolysis is comparable; (ii) on the other hand, the planarity of the cyclophosphazene ring—at least in the trimers $N_3P_3X_6$—could confer intercalating properties on the cyclophosphazenes.

This paper reports on the antitumour tests. Six cyclophosphazenes, namely the hexachlorocyclotriphosphazene $N_3P_3Cl_6$, the hexaziridinocyclotriphosphazene $N_3P_3Az_6$, the hexapyrrolidinocyclotriphosphazene $N_3P_3Pyrro_6$, the octachlorocyclotetraphosphazene $N_4P_4Cl_8$, the octaziridinocyclotetraphosphazene $N_4P_4Az_8$ and the octapyrrolidinotetracyclophosphazene $N_4P_4Pyrro_8$ were tested on mouse P388 and L1210 leukemias and B16 melanoma.

MATERIALS AND METHODS

1. *Synthesis and purity*

$N_3P_3Cl_6$ was obtained from Fine Chemicals (purity > 93%). $N_4P_4Cl_8$ was obtained from R. A. Shaw (Birkbeck College, London) to whom we are indebted for his generosity. These starting materials were recrystallized at least 6 times from acetonitrile ($N_3P_3Cl_6$) and from petroleum ether (60–80°C) ($N_4P_4Cl_8$). *n*-Hexane should be avoided as crystallizing solvent since $N_3P_3Cl_6$ which exhibited arrangement therein, leading to $N_4P_4Cl_8$, $N_5P_5Cl_{10}$ and higher isologs, as demonstrated

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by ^{31}P NMR spectroscopy ($\delta^{31}P = -19.5$, $+8.0$, $+17.0$ and $+19.5$ ppm respectively for $N_3P_3Cl_6$, $N_4P_4Cl_8$, $N_5P_5Cl_{10}$ and higher isologs with 85% H_3PO_4 as a standard).

The two trimers, $N_3P_3Az_6$ and $N_3P_3Pyrro_6$, were prepared using Rätz's procedure [10] in the presence of ammonia; the two tetramers, $N_4P_4Az_8$ and $N_4P_4Pyrro_8$, were obtained by the same process but in the presence of triethylamine.

2. *Solutions*

Since all derivatives studied, except $N_3P_3Az_6$, had a very poor solubility in water, they were inoculated as suspensions in 4‰ hydroxypropylcellulose (Klucel J. F., Hercules Co) solutions in water. Such Klucel solutions were shown to be non-toxic after i.p. inoculation. Moreover, the size of the molecular aggregates in such Klucel solutions was much less than 1 μm, as demonstrated by electron microscopy.

$N_3P_3Az_6$ on the contrary is highly soluble in water, about 100 g/l, and could consequently be used in 0.9% NaCl water solution as for both the i.p. and the i.v. route.

ANIMAL STUDIES

1. *Toxicity measurements*

The toxicity of the drugs was determined either on female swiss or on female DBA/2 mice. The lethality—which happens systematically on days 5–6 after administration—was recorded as a function of the dose inoculated and there allowed to deduce the LD_0 values which correspond to the highest non-lethal doses. The results do not depend on the species of mice we used.

2. *Antitumour tests*

The L1210 and P388 cells were maintained by weekly passages (i.p. inoculation) of ascites cells in female DBA/2 mice (Centre de Sélection et d'Elevage des Animaux de Laboratoires du CNRS, Orléans-La Source, France). The B16 cells were maintained by 10–14 days passages (s.c. inoculation) of solid tumor cells in female C57 Black mice (same origin). Experiments were conducted on mice weighing (20 ± 2) g which were about 2 months old (same origin). Fifteen mice were used per group and the deaths were recorded daily at the same hour.

The mean survival times of the treated mice (T) and of the control (C) were used to calculate the percentage increase in median life time over control

$$\%\text{ILS} = \frac{T-C}{C} \cdot 100$$

which is significant for antitumour activity only when higher than 25%.

For B16 melanoma, the median diameter of the tumours on the 12th and on the 14th day after the tumour graft for the control and treated sets of mice were measured: this blind procedure allows indeed to appreciate the way in which a drug inhibits the growing of a solid tumor.

The antitumour tests were performed using the standard NCI protocols [11]: the leukemia or the melanoma being transplanted on the day D, the drug was inoculated by i.p. route (except for $N_3P_3Az_6$ where i.p. and i.v. routes could be used) on the day $D+1$ (monoinjection protocol) or within a QnD schedule.

RESULTS

1. *Toxicity*

The LD_0 values obtained for the six cyclophosphazenes studied are given in Table 1. Two points may be emphasized:

(a) For a given substituent X, the trimer $N_3P_3X_6$ and the corresponding tetramer $N_4P_4X_8$ have practically the same LD_0 value, except in the case $X=Cl$ where the trimer $N_3P_3Cl_6$ appears to be about 15 times more toxic than its tetrameric isologue.

(b) For $N_3P_3Az_6$, the i.p. and i.v. LD_0 are quite similar, about 40 mg/kg.

Table 1. *Highest non-lethal doses* LD_0 *for the* 6 *cyclophosphazenes studied*

Compound	LD_0 (mg/kg)
$N_3P_3Cl_6$	20
$N_3P_3Az_6$	40*
$N_3P_3Pyrro_6$	10
$N_4P_4Cl_8$	300
$N_4P_4Az_8$	75
$N_4P_4Pyrro_8$	20

*Both for i.p. and i.v. routes.

2. *Antitumour activity*

(*a*) *Effects on the P388 leukemia.* In Table 2 are shown the activities of $N_3P_3Az_6$, $N_4P_4Az_8$

Table 2. Antitumoral activity of cyclophosphazenes against P 388 leukemia

Compound	Schedule	Dose (mg/kg/day)	%ILS
	Once, day 1 (i.p.)	2.5	21
		10	51
		20	101
$N_3P_3Az_6$	Q4D; days 1,5,9	10	100
($LD_0 = 40$ mg/kg)	Once, day 1 (i.v.)	10	17
		20	47
		30	69
	Once, day 1 (i.p.)	10	18
		20	47
$N_4P_4Az_8$		30	49
($LD_0 = 75$ mg/kg)		40	54
		50	57
	Q4D; days 1,5,9	40	101
$N_4P_4Pyrro_8$	Once, day 1 (i.p.)	5	24
($LD_0 = 20$ mg/kg)		10	33

10^6 P388 cells implanted i.p., i.p. or i.v. treatment (15 mice per group); $N_3P_3Az_6$ was dissolved in 0.9% NaCl solution; $N_4P_4Az_8$ and $N_4P_4Pyrro_8$ were suspended in 4‰ Klucel JF (Hercules Co.) water solution; median survival time of control: 9.9 days.

and $N_4P_4Pyrro_8$ under various conditions. The three other cyclophosphazenes, including $N_3P_3Cl_6$, were found to be non-significantly active (i.e., $\%ILS \leqq 25$) in our experimental conditions.

Moreover, the following points may be emphasized:

(1) $N_3P_3Az_6$ appears to be the most active member of the series: indeed a dose of 2.5 mg/kg leads to an ILS value which approaches the 25% level whereas an injection of 20 mg/kg enhances the ILS value to 101% (with, however, 3/15 mice dying on days 5–6). It may be noted that an i.p. dose of 40 mg/kg led to an ILS equal to 166% (not including 2 cured mice) but the mortality (6/15) in such conditions could not be considered as acceptable.

The therapeutic index of $N_3P_3Az_6$, defined as the ratio of the LD_0 value divided by the dose which gives an ILS of 40%, is about 6.

(2) The use of the Q4D [1, 5, 9] schedule noticeably increases ILS figures with respect to the monoinjection D + 1 protocol: the ILS value is multiplied by a factor 2 indeed when passing from a (1.10) injection to a Q4D (3.10) one. A factor 2 is also observed for $N_4P_4Az_8$ when passing from a (1.40) injection to the Q4D (3.40) one.

(3) Figure 2 shows the linear dose–activity relationships for $N_3P_3Az_6$ by i.p. and i.v. routes. The i.v. route affords ILS values of about 70% without side-toxicity. It may be noticed that an ILS value of 91% was obtained for an i.v. dose equal to 40 mg/kg but the accompanying side-toxicity (7/15) was not acceptable.

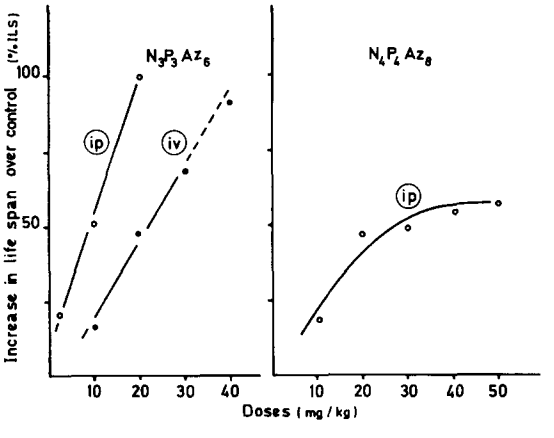


Fig. 2. Activity–dose relationships for $N_3P_3Az_6$ and $N_4P_4Az_8$ on P388 leukemia.

(4) The dose–activity relationship for $N_4P_4Az_8$ in a monoinjection protocol by i.p. route (Fig. 2) in Klucel appears to be linear between 10 and 20 mg/kg, a levelling-off trend occurring for higher doses without any significant side-toxicity. The therapeutic index of $N_4P_4Az_8$ (as defined above) is *ca.* 4.

From the foregoing results, $N_3P_3Az_6$ seems the most promising antitumour agent of the series tested against the P388 leukemia and has the additional advantage of a high solubility in water and of being active both by the i.p. and the i.v. route.

(*b*) *Effects on the L1210 leukemia.* The tests on L1210 leukemia (and on B16 melanoma) were confined to the members of the series which exhibited a significant activity on the P388 tumour.

The activities for $N_3P_3Az_6$ and $N_4P_4Az_8$ under various conditions using the i.p. route are shown in Table 3. $N_4P_4Pyrro_8$ was found to be non-significantly active.

leukemias. From Table 4, $N_3P_3Az_6$ is the only cyclophosphazene giving ILS approaching or exceeding the 40% level, either using a $D+1$ monoinjection (1.40) schedule of treatment or within a Q3D [1, 4, 7, 10, 13] (5.20) schedule. Furthermore, the quantity of the first inoculation (i.e., 40 mg/kg on the $D+1$ day) seems to be determinant, the ILS value (53%) being larger than that (39%) obtained with the Q3D schedule.

For some of these treatment schedules (Table 5) both the number of tumour-bearing mice and the tumour diameters on the 12th and on the 14th day were recorded: When compared to the control, the number and the

Table 3. *Antitumoral activity of cyclophosphazenes against L1210 leukemia*

Compound	Schedule	Dose (mg/kg/day)	%ILS
$N_3P_3Az_6$ ($LD_0 = 40$ mg/kg)	Once, day 1	10	28
		20	45
	Q3D; days 1,4,7	10	44
$N_4P_4Az_8$ ($LD_0 = 75$ mg/kg)	Once, day 1	40	8
		50	22
		60	17
	Q4D; days 1,5	40	20
	Q3D; days 1,4,7	40	44
$N_4P_4Pyrro_8$ ($LD_0 = 20$ mg/kg)	Once, day 1	5	9

10^5 L1210 cells implanted i.p., i.p. treatment (15 mice per group); $N_3P_3Az_6$ was dissolved in 0.9% NaCl solution; $N_4P_4Az_8$ and $N_4P_4Pyrro_8$ were suspended in 4‰ Klucel JF (Hercules Co.) water solution; median survival time of control: 8.5 days.

ILS figures were definitely smaller for the L1210 than for the P388 tumour: $N_3P_3Az_6$ is the only compound which exhibits a significant (i.e., %ILS > 25) activity in a monoinjection protocol. A Q3D [1, 4, 7] schedule chosen in order to take into account the fact that the median survival time of control animals is only about 8.5 days for L1210 vs 9.9 days for P388—was used to get significant ILS values for $N_4P_4Az_8$.

However, as with the P388 tumour, ILS figures are approximately 2-fold greater, for a given dose, using the Q3D polyinjection schedule compared with the $D+1$ monoinjection protocol.

(*c*) *Effects on the B16 melanoma.* B16 melanotic melanoma is a slow-growing tumour when compared to the L1210 and P388 size of tumours for the treated mice was considerably smaller, indicating a real effectiveness for the drugs against B16 melanoma. Moreover, these two parameters are in good accord with the ILS determinations.

DISCUSSION

Amongst the six cyclophosphazenes studied, three, namely $N_3P_3Az_6$, $N_4P_4Az_8$ and $N_4P_4Pyrro_8$, exhibited a significant antitumour activity on P388 leukemia on i.p. administration. The most active is $N_3P_3Az_6$ which increases by a factor of 2 the median survival time of treated animals compared with controls (i.e., %ILS ~ 100) either within an i.p. monoinjection protocol (1.20 mg/kg) or within an i.p. Q4D schedule (3.10 mg/kg).

Table 4. Antitumoral activity of cyclophosphazenes against B16 melanoma

Compound	Schedule	Dose (mg/kg/day)	%ILS
	Once, day 1	30	17
		40	53
$N_3P_3Az_6$	Q3D; days 1,4,7,10,13	10	22
($LD_0 = 40$ mg/kg)	Q4D; days 1,5,9	20	28
	Q3D; days 1,4,7,10,13	20	39
$N_4P_4Az_8$	Once, day 1	50	10
($LD_0 = 75$ mg/kg)	Q4D; days 1,5,9	40	30
$N_4P_4Pyrro_8$	Q4D; days 1,5,9	10	10
($LD_0 = 20$ mg/kg)			

B16 cells implanted s.c., i.p. treatment (15 mice per group): $N_3P_3Az_6$ was dissolved in 0.9% NaCl solution; $N_4P_4Az_8$ and $N_4P_4Pyrro_8$ were suspended in 4‰ Klucel JF (Hercules Co.) water solution; median survival time of control: 22.0 days.

Table 5. Comparative effects of some cyclophosphazenes on B16 tumor evolution

	12th day		14th day		
Compound and schedule	Number of tumor-bearing mice	Size of tumors (cm)*	Number of tumor-bearing mice	Size of tumors (cm)*	%ILS
Control	14/15	1.2±0.2	15/15	1.4±0.1	—
$N_3P_3Az_6$					
(1.40)	5/13	0.4±0.2	7/13	0.6±0.2	53
(5.10)	10/15	0.8±0.2†	10/15	1.0±0.2	22
(5.20)	6/14	0.5±0.2	8/14	0.5±0.1	39
$N_4P_4Az_8$					
(3.40)	9/15	0.6±0.2	11/15	0.7±0.2	30
$N_4P_4Pyrro_8$					
(3.10)	9/13	0.6±0.1	12/13	1.0±0.1	10

*Mean ±S.E. of the mean, calculated on the total number of mice per group (a non-tumored mouse was counted as zero but involved into the calculation). The difference in median size of treated and control series were statistically significant (Student's *t*-test) at $P<0.05$ unless for † where $P<0.10$ only.

Furthermore, this derivative has the advantage of high water-solubility and of activity also by the i.v. route: a single dose of 30 mg/kg afforded an ILS of 69%. The ratio of the LD_0 value to the minimum significantly active dose is about 16 after i.p. administration which is amongst the highest values ever observed for antitumour drugs.

Moreover, $N_3P_3Az_6$ shows a linear dose–activity relationship either by the i.p. or i.v. route (Fig. 2) whereas for $N_4P_4Az_8$ there is a levelling-off effect which may be due to a compartment effect analogous to that investigated by Le Pecq *et al.* [12] for 9-hydroxyellipticine.

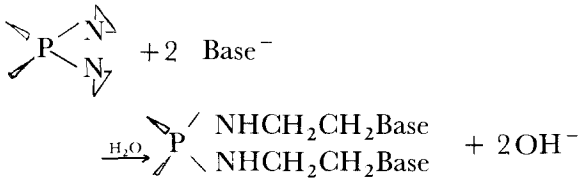
Against the L1210 leukemia, only two cyclophosphazenes, namely $N_3P_3Az_6$ and $N_4P_4Az_8$, were active, the first affording to a significant ILS value even within a monoinjection protocol whereas a Q3D schedule is required to obtain an ILS larger than 25% for the latter.

The same conclusions broadly apply to the B16 melanoma results: the optimal dose is 1.40 mg/kg (%ILS = 53) for $N_3P_3Az_6$ and 3.40 mg/kg (%ILS = 30) for $N_4P_4Az_8$. The additional check on the number of tumour-bearing mice and of tumour diameters on the 12th and on the 14th day after the graft have shown that tumour growth is significantly delayed by the active doses just mentioned (Table 5).

The main conclusion from these tests on the antitumour activities of six cyclophosphazenes against P388, L1210 and B16 tumours is that the most effective was in each case the hexaziridinocyclotriphosphazene $N_3P_3Az_6$ whereas the molecule on which we had based the

theoretical concepts behind our work, namely $N_3P_3Cl_6$, seems not to be active, at least under our experimental conditions. Such a result requires some comments in the light of the ideas developed in the early part of this paper:

1. $N_3P_3Az_6$ is a molecule in which the planar N_3P_3 ring carries *pairs* of aziridino groups* which may act as bifunctional alkylating agents of DNA by way of the classical reaction



With regard to the relative antitumour activities of $N_3P_3Az_6$ and of $N_3P_3Cl_6$, aziridino groups seem consequently to be more active in that respect than chlorine atoms when substituted on a cyclophosphazene ring.

2. $N_3P_3Az_6$, containing aziridino groups, may belong to the same class of antitumour agents as Thiotepa ($SPAz_3$), TEM (trisaziridinomelamine, $N_3C_3Az_3$) or aziridinylbenzoquinones [13], i.e. alkylating antitumour agents. We did not repeat any previous experiment which had been performed about these drugs but looking at Skipper's monograph [14], it is reported that Thiotepa is non-active—in contrast to $N_3P_3Az_6$—on the early L1210 leukemia implanted either by i.p. or by s.c. route, being active only on the advanced s.c. L1210 leukemia; $N_3C_3Az_3$ (TEM) has practically the same behaviour, being however active on the early L1210 leukemia implanted by s.c. route. Thus, the effectiveness of $N_3P_3Az_6$ on early leukemias appears superior than that of Thiotepa or of TEM. In seeking an explanation for this fact, the target site and the mode of action of the drug in question is currently being investigated.

Ames tests (P. Lecointe, to be published) and a general study of the DNA-cyclophosphazene complexes by fluorescence using the ethidium bromide as a probe [15] are now in progress in our laboratory: the preliminary results support the dialkylating action on DNA we mentioned above and Lecointe was able to show that $N_3P_3Az_6$ is equally mutagenic on TA 100 and 1535 strains either with or without metabolism. This result encourages us to search for an eventual connecting relationship between the X-ray crystal structures *in vitro* ([16] and in progress) of the studied cyclophosphazenes and their antitumour activities *in vivo*.

The synthesis of new aziridino cyclophosphazene analogues designed to have a higher dialkylating activity towards the DNA and perhaps, as a consequence, a higher antitumour activity is currently being carried out in our laboratory.

*Incidentally, we controlled that aziridine itself has no activity at all *per se* on P388, even within a QnD schedule.

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