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Inorganic Ring Systems

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Table of Contents

Up-to-date Improvements in Inorganic Ring Systems as Anticancer Agents[1]

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Tables of Contents

[1] Plenary lecture delivered at the 3rd IRIS Meeting held in GRAZ (Austria), August 17–22, 1981

1 Introduction

Cancer does not exist. There exist actually 104 different types of cancers — or more precisely of *human tumors* — which may attack any part of the body and which nearly always enter by furtive and mysterious routes, depending on so many intangible factors, that mankind has up to now been completely powerless to prevent cancerisation in humans.

Thus, the only possible way to escape from the terrifying corners into which cancers are every day driving more and more humans is, of course, to detect them as early as possible and to start immediately the appropriate treatments, if any.

Unfortunately, the reality of the situation is not so simple. However large the arsenal of weapons that clinicians may have at their disposal, there are more than 40 kinds of tumors that cannot be cured at all nowadays by any medical approach. Thus, for such tumors, diagnosis means unstoppable slides to death. The situation is scarcely better for about 40 other tumors, mainly when they are so spread out over the whole body that surgery and radiotherapy cannot be used, so that chemotherapy and immunotherapy have to be employed.

In other words, in spite of the efforts performed and the relative successes obtained since the end of the 2nd world war within the field of this struggle for life, i.e., against cancers, we are still in the Stone Age. Thus, any new idea, approach or concept, even apparently crazy or fully outside the scope of the well-established dogmas, must be examined, criticized and tried out.

2 The Specific Role of Chemotherapy Amongst the Anticancer Weapons

Sword (surgery), artillery (radiotherapy), asphyxiating gases[2] (chemotherapy) and jiu-jitsu or aïki-do (immunotherapy) are the four weapons against cancers. They must be used in concert in any clinical treatment of localized tumors which can be concomitantly excised, irradiated and/or size-reduced by a drug.

However, as we mentioned above, chemotherapy and immunotherapy are still the only weapons applicable either when solid tumors are delocalized over a large area of the body or when the tumors are liquid (ascites tumors like leukemias).

Thus, chemotherapy must be considered as a curative technique which may be of vital help for any kind of tumor. Such a privileged role appears a bit surprising if we remember that about 60% of human cancers are generally assumed, according to world Health Office Statistics, to be induced by our chemical environment (tobacco, cosmetics, food dyes, nitrosamines, . . .). Consequently, treating cancers by chemistry looks a priori paradoxical. However, one may understand that some chemists have to be under the obligation to repair damage created by other chemists, so mueh the more that the percentage of chemists who become cancerous is about 25% larger than the one which is observed for non chemists.

[2] The first really efficient anticancer drugs were indeed "nitrogen mustards", derived from yperite, one of the notorious poison gases of the 1st World War.

3 How to Design an Anticancer Drug?

When surveying the literature on this subject over the last thirty years, we may notice that pathways for such a discovery were essentially as follows.

(i) About 80% of the drugs used to-day at the clinical human level were detected through the huge systematic screening developed by NCI (National Cancer Institute) in the States during the 1950's. Within the last three decades NCI has tested more than 800,000 chemicals on probably billions of tumor-bearing mice and rats. This "blindeyes" investigation has yielded about 30 effective drugs against several types of human tumors. The incredibly small ratio of the two previous figures may stupefy beotians. However, this somewhat desperate method has provided many powerful anticancer drugs which would have never been detected by any other, pseudo-logical, approach.

(ii) The discovery by Barnett Rosenberg of many very active platinum drugs started from a shrewd observation of an unexpected experiment: mitosis of cells in NH_4Cl buffer solution appeared to be deeply inhibited when subjected to an electric field produced by two "inert" (!) platinum electrodes ... Rosenberg could have concluded that the electric field was the inhibiting factor. But he did not fall into this trap; rather he demonstrated excellently that such an inhibition was actually due to some $(NH_3)_2PtCl_2$ entity produced by chemical reaction between NH_4Cl and the so-called inert Pt electrodes. The first cis-platinum drug was discovered in this way and everybody knows how fruitful this lucky find has been for further treatment of many cancers.

(iii) The antitumoral properties of some plants (roots, stems, leaves) and fungi were intuitively known by many tribes or peoples, sometimes for several centuries. For example, in 1609 a Dutch clinician reported at the end of a medical trip to Moluccas Islands that natives were successfully treating cancer of the nostrils by repeated applications of ground roots of a local plant called *Elliptica*. More than three centuries later, some Australian researchers prepared some anticancer alkaloïds derived from a molecule they called Ellipticine, owing to the fact that this chemical had been demonstrated as being actually the active principle of the Moluccan elliptica. Incidentally, ellipticine was also extracted from some other plants, i.e., apocynaceae (ochrosia moorei and excavatia coccinea).

In other words, several anticancer drugs are present in nature and they may be isolated by appropriate chemical techniques. The yield of such extractions, however, remains obviously very low and chemists are normally required to prepare synthetically large quantities of these natural products.

Other examples of natural drugs may be pointed out: streptozotocin (from streptomyces achromogenes), bleomycin A_2 (from streptomyces verticillus), adriamycin and daunomycin (from streptomyces pencetius), mitomycin C (from streptomyces caesipitosus), vincristine, vinblastine and vindoline (from catharanthus roseus or vinca rosea L.).

(iv) In contrast with the previous approaches, one may envisage a more logical route based on some structural molecular peculiarities which would be common to several individual drugs or series of drugs.

Let us consider for example the geometrical structure of Rosenberg's active platinum drugs. Up to now, the most efficient anticancer derivatives of the series have

a square-planar Pt (II) uncharged cis-structure (Fig. 1). The activity of such drugs was demonstrated as occurring through a *dialkylation* of DNA on N7 and O6 sites of guanine (Fig. 2) by the mean of the two labile Cl atoms of the molecule. Rosenberg claimed that the distance (3.4 Å) [1] between these chlorines may be considered as a "magic number" for suitable strong dialkylation of DNA.

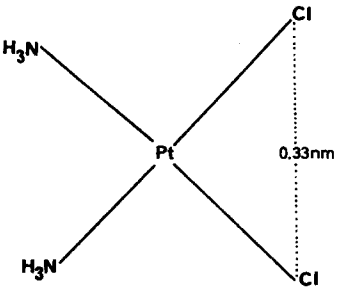

Fig. 1. Geometry of Rosenberg's cis-platinum

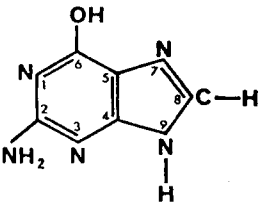

Fig. 2. Numbering of atoms in Guanine

Let us now consider some other anticancer drugs such as ellipticines or adriamycin, which prevent the replication of vicious DNA through a process of *intercalation* between plates (A ... T and G ... C) of bases. The effectiveness of these drugs seems related to the plus or minus chemical stability of such an intercalation and this stability is generally determined by strong hydrogen bonds between endocyclic nitrogen atoms or molecular oxygen atoms with sites on the ribose backbone of DNA. This binding mechanism obviously depends on the basicity of the N and O atoms in question: the larger the basicity, the stronger the stability of interaction and, consequently, the higher the effectiveness.

Thus, if a molecular structure contains both (i) pairs of chlorine atoms in a "square-planar-like" 3.4 Å situation and (ii) a planar ring with highly basic endocyclic N or O atoms, we may expect that the coexistence of these two structural peculiarities will confer a potential antitumor activity on the molecules in question.

This is actually the case with hexachlorocyclophosphazene (Fig. 3) and relatives and this is the starting idea of the investigations, which we began in 1976, on the application of cyclophosphazenes as anticancer agents.

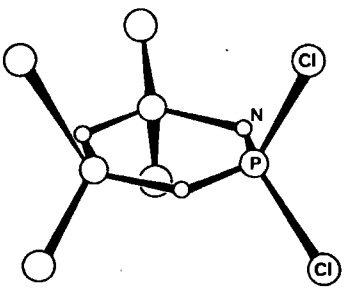

Fig. 3. Geometrical structure of $N_3P_3Cl_6$

4 Cyclophosphazenes as Anticancer Agents

The first investigations on the antitumor activity in vivo of cyclophosphazenes were started late in 1976 on murine L1210 and P388 leukemias (DBA/2 female mice) and on sub-cutaneous (s.c.) B16 melanoma (C57 black female mice).

For the first step, we chose 8 cyclophosphazenic systems containing either chlorine pairs (Rosenberg magic number) or highly basic endocyclic nitrogen atoms (intercalation process), namely $N_3P_3Cl_6$ (I), $N_4P_4Cl_8$ (II), $N_3P_3Az_6$ (III), $N_4P_4Az_8$ (IV), $N_3P_3Pyrro_6$ (V), $N_4P_4Pyrro_8$ (VI), $N_3P_3Morph_6$ (VII) and $N_4P_4Morph_8$ (VIII) (Az = Aziridinyl; Pyrro = Pyrrolidinyl; Morph = Morpholinyl).

4.1 Synthesis and Purity

(I) was obtained at that time from Fine Chemicals (purity > 93%), (II) was obtained from R. A. Shaw (Birkbeck College, London) to whom we are indebted for this generosity. These materials were recrystallized at least 6 times from acetonitrile for (I) and from petroleum ether (60–80 °C) for (II). n—Hexane should be avoided as crystallizing solvent since (I) exhibited arrangement therein, leading to (II), $N_5P_5Cl_{10}$ and higher isologs, as demonstrated by ^{31}P NMR spectroscopy ($\delta^{31}P$ = −19.5, +8.0, +17.0 and +19.5 ppm respectively for (I), (II), $N_5P_5Cl_{10}$ and higher isologs with 85% H_3PO_4 as a standard).

The three trimers, (III), (V) and (VII), were prepared using Rätz's procedure [2] in the presence of ammonia; the tetramers, (IV), (VI) and (VIII), were obtained by the same process but in the presence of triethylamine.

4.2 Solutions

Since all the derivatives studied, except (III), had a very poor solubility in water (<2 g/l), 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. (intra-peritoneal) inoculation. Moreover, the size of the molecular aggregates in such Klucel solution was much less than 1 μm, as demonstrated by electron microscopy.

(III) on the contrary is highly soluble (without any hydrolysis) in water, about 100 g/l, and could consequently be used in 0.9% aqueous NaCl solution (saline) as for both the i.p. and the i.v. (intraveinous) route.

4.3 Toxicity Measurements

The toxicity of the drugs was determined either on female Swiss or on female DBA/2 mice. The lethality — which happens systematically 5–6 days after administration — was recorded as a function of the dose inoculated and 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.

LD_0 values are gathered in Table 1. Two points may be emphasized.

(i) 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 (I) appears to be about 15 times more toxic than its tetrameric isolog (II).

(ii) For (III) 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 8 cyclophosphazenes studied. (in mg/kg)

$N_3P_3Az_6$	(III)	40
$N_4P_4Az_8$	(IV)	75
$N_3P_3Pyrro_6$	(V)	10
$N_4P_4Pyrro_8$	(VI)	20
$N_3P_3Morph_6$	(VII)	150
$N_4P_4Morph_8$	(VIII)	>600
$N_3P_3Cl_6$	(I)	20
$N_4P_4Cl_8$	(II)	300

4.4 Antitumor Tests

The L1210 and P388 cells were maintained by weekly passages (i.p. inoculation) of ascites cells in female DBA/2 mice (Centre de Selection 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. 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 antitumor activity only when higher than 25%.

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

The antitumor tests were performed using the standard NCI protocols [3]: the leukemia or the melanoma was transplanted on the day D, and the drug was inoculated by i.p. route (except for (III) where i.p. and i.v. routes could be used) on the day D + 1 (monoinjection protocol) or within a QnD schedule (the same dose being injected at intervals of n days from the day D + 1).

4.5 Antitumor Activity of Compounds (I) to (VIII) [4)]

4.5.1 Effects on the P388 Leukemia

In Table 2 are shown the activities of (III), (IV) and (VI) under various conditions. The five other cyclophosphazenes, including the chlorine derivatives (I) and (II), were indeed found to be just at the limit of a significant activity (i.e., % ILS ~25) within heavy Q4D (1, 5, 9, 13, 17) schedules. In other words, it is clear that the magic number assumption does not work as well as we could have expected; however, $N_3P_3Cl_6$ and $N_4P_4Cl_8$ must be considered as antitumor agents on P388, even if a repeated long polyinjection schedule is required to make their effectiveness conspicuous.

Table 2. Antitumor activity of some cyclophosphazenes against P388 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.

From Table 2, the following points may be emphasized.

(i) (III) appears to be the most active member of the series: indeed a single dose of 2.5 mg/kg leads to an ILS value which approaches the 25% level whereas an injection of 20 mg/kg ($LD_0/2$) enhances the ILS value to 101%. It may be noted that an i.p. dose of 40 mg/kg (LD_0) leads to an ILS equal to 166% (*not including 2 cured mice*) but the acute mortality (6 mice over 15 on days 5–6 after administration) under such conditions could not be considered as acceptable.

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

(ii) 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 indeed multiplied by a factor 2 when passing from a (1.10) injection to a Q4D (3.10) one. A factor of 2 is also observed for (IV) when passing from a (1.40) injection to the Q4D (3.40) one.

(iii) Figure 4 shows the linear dose-activity relationships for (III) by i.p. and i.v. routes. The i.v. route affords ILS values of about 70% in monoinjection 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 (6 mice over 15 on days 5–6) was not acceptable.

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

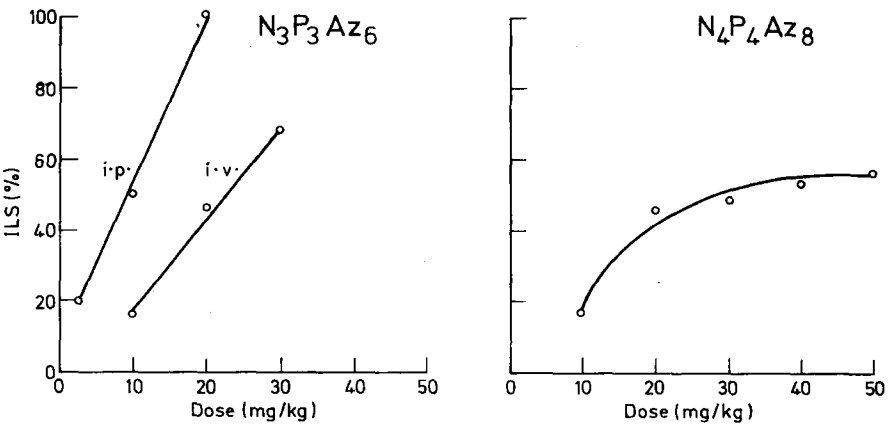

Fig. 4. Activity-dose relationships for $N_3P_3Az_6$ and $N_4P_4Az_8$ on P388 leukemia

From the foregoing results, $N_3P_3Az_6$ seemed the most promising antitumor 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.

4.5.2 Effects on the L1210 Leukemia

The tests on L1210 leukemia (as well as on B16 melanoma) were confined to the members of the series which exhibited a significant activity on the P388 tumor.

The activities for (III) and (IV) under various conditions using the i.p. route are shown in Table 3. (VI) was found to be non-significantly active, even within a Q3D (1, 4, 7) schedule.

ILS Figs. are definitely smaller for the L1210 than for the P388: (III) 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 L 1210 *vs* 9.9 days for P388 — has to be used to get significant ILS values for (IV).

However, as with the P388 tumor, ILS figures are approximately twice as large, for a given dose, using the Q3D polyinjection schedule compared with the D + 1 monoinjection protocol.

Table 3. Antitumor activity of some cyclophosphazenes against 1210 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 L 1210 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.

Table 4. Antitumor activity of some cyclophosphazenes against B16 sub-cutaneous melanoma

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

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.

4.5.3 Effects on the B16 Sub-Cutaneous Melanoma

B16 sub-cutaneous melanoma is a slow-growing tumor when compared to the L1210 and P388 leukemias. This is a very tedious tumor on which very few drugs were found to be significantly active, (i.e., % ILS > 40%).

From Table 4, (III) is the only cyclophosphazene giving ILS approaching or exceeding the 40% level, either using a D + 1 monoinjection (1.40) protocol 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 day D + 1) 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 tumor-bearing mice and the tumor diameters on the 12th and on the 14th day were recorded: when compared to the control, the number and the size of tumors for the treated mice were considerably smaller, indicating the drugs were genuinely effective against s.c. B16 melanoma. Moreover, these two parameters are in good accord with the ILS determinations.

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

12th day			14th day		
Compound and schedule	Number of tumor-bearing mice	Size of tumors (cm)[a]	Number of tumor-bearing mice	Size of tumors (cm)[a]	%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

a 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.

In conclusion of these antitumor tests on murine L1210, P388 and B16 tumors, it might be pointed out that the most effective in each case was the hexaziridino-cyclotriphosphazene $N_3P_3Az_6$ whereas $N_3P_3Cl_6$ and $N_4P_4Cl_8$ appear to be poorly active, at least under our experimental conditions. Such a result requires some comments in the light of the ideas developed above.

(i) $N_3P_3Az_6$ 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