Inorganica Chimica Acta, 108 (1985) 23–27

Covalent Binding of Non-effective Diaziridinocyclotriphosphazenes to Natural Polyamines as Tumor Finders makes Potential Anticancer Agents

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Received March 21, 1985

Abstract

Covalent binding of diaziridinocyclotriphosphazenes, reputed as non-effective antitumor agents, to natural polyamines as tumor finders gives vectorized diaziridinocyclotriphosphazenes which display a significant effectiveness on murine tumors *in vivo*.

Introduction

The possibility that certain phosphonitrile polymers might have antitumor activity was first studied in 1959 [1] but the investigations attracted little attention at the time. Interest was revived when a new series of aziridinocyclophosphazenes were examined [2] and the hexaziridino trimer $N_3P_3Az_6$ proved to be active against various experimental tumors [3]. More recently, developments in the study of the inorganic thiatriazadiphosphorine ring compounds have shown that the pentaziridino derivative, designated for convenience as SOAz [4], has an interesting degree of antitumor activity and is, globally, of lower toxicity than $N_3P_3Az_6$ [5–7].

In current studies, it has also been found that a reduction from 6 to 4 in the number of Az ligands grafted on the N_3P_3 ring, given that $N_3P_3Az_5Cl$ and gem-$N_3P_3Az_4Cl_2$ (coded as MYCLAz), produces compounds which possess a still significant antitumor activity, more or less equal to $N_3P_3Az_6$ [8, 9]. In contrast, when the number of Az ligands on the N_3P_3 ring drops to 2, the antitumor effectiveness is no longer observed, whatever the four other ligands [1, 10].

However, pharmacokinetics has proved that 60% of SOAz injected into animals and/or humans [11] is excreted through urine (without any metabolisation) during the proceeding 24 h. In other words, a large part of the remedy does not reach the tumor and is distributed all over the body without any therapeutical effect, thus inducing side-effects.

Therefore, in trying to increase selectivity for malignant cells of SOAz and relatives and to decrease their toxicity for normal tissues, it was necessary to investigate the activity of antitumor cyclophosphazenes when linked to natural polyamines. It is well-known that rapidly proliferating cells have a higher capactity for active, carrier-mediated uptake of natural polyamines [12, 13]. Therefore, natural polyamines could represent a useful means to aim at neoplastic tissue compounds possessing cell-inhibitory capacity.

This attempt to produce more selective antitumor cyclophosphazenes was successful in the case of MYCLAz when covalently bound to 1,3-diaminopropane and putrescine in a SPIRO configuration [14–16]: these vectorized super-drugs, coded as DIAM3 and DIAM4 for 1,3-diaminopropane and putrescine as a tumor finder, respectively, are about 5 times less toxic than 'nude' MYCLAz when their therapeutic index becomes 10 to 15, *vs.* 4 for MYCLAz alone. Further trials to produce other more selective antitumor cyclophosphazenes by means of spermidine and spermine as tumor finders are now in progress.

Thus, the antitumor effectiveness of active aziridinocyclotriphosphazenes and relatives derives its benefit from a covalent binding to natural polyamines. Incidentally, it is noteworthy that such binding occurs in a stereo-selective, stereo-specific and regio-specific chemical manner [17], providing the chemist with an interesting box of bricks which we labelled as 'BASIC', *i.e.* 'BINO-ANSA-SPIRO IN CYCLOPHOSPHAZENES' [18].

To revert to the so-called non-effectiveness of the 'nude' diaziridinocyclotriphosphazenes we mentioned above, the following question now arises: (i) either such non-effectiveness is an intrinsic property due to the lack of Az ligands on the N_3P_3 ring (ii) or it is due to the very poor bio-availability of molecules, essentially because of their very low water solubility.

In other words, we wondered whether a covalent binding of such non-active diaziridinocyclotriphos-

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0020-1693/85/$3.30

phazenes to some natural polyamines as tumor finders would not provide super-molecules which would be highly water soluble and would, consequently, display significant antitumor activity.

This report describes the synthesis, physicochemical identity and biological activity *in vivo* of the super-molecule type **(I)** which is visualized in Fig. 1.

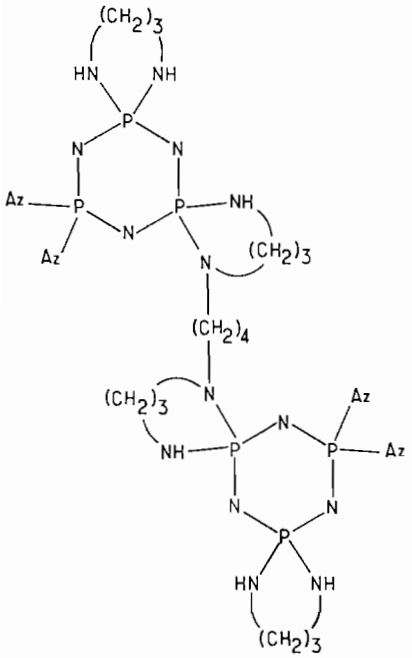

Fig. 1. Chemical formula of the super-drug **(I)**.

Experimental

Synthesis, Identification and Purity of (I)

Compound **(I)** was synthesized through the following three-step path:

(1) Reaction of 1,3-diaminopropane with $N_3P_3Cl_6$ in stoichiometric conditions, leading selectively to the SPIRO–$N_3P_3Cl_4$ [HN–$(CH_2)_3$–NH] derivative (hereinafter coded as SPIRO) [19, 20];

(2) Reaction of SPIRO with spermine H_2N–$(CH_2)_3$–NH–$(CH_2)_4$–NH–$(CH_2)_3$–NH_2 in (2:1) conditions, leading to the two-ring assembly structure (hereafter described as SPMCl) in which two SPIRO–$N_3P_3Cl_2$ [HN–$(CH_2)_3$–NH] moieties are bridged through a spermino entity in a DISPIROBINO [21] configuration (Fig. 2);

(3) Peraziridinolysis of SMPCl, leading to the expected final product **(I)**.

The synthesis of SPIRO was previously described [19]. Thus, herein, we only discuss the synthesis of SMPCl and of **(I)**.

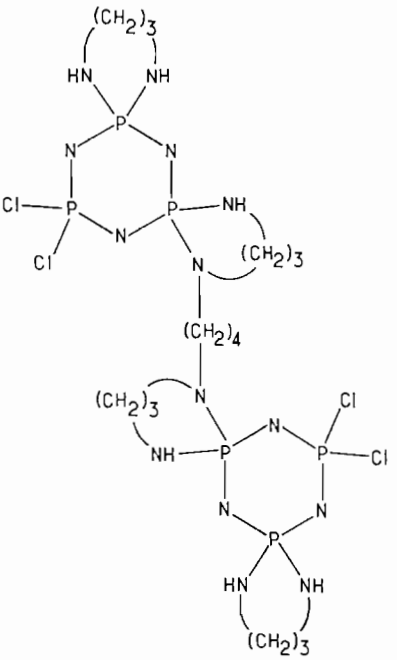

Fig. 2. Chemical formula of SPMCl.

For SPMCl, 6 g of spermine in 300 ml of CH_2Cl_2 were added dropwise in 1 h to a mixture of 20.6 g of SPIRO and 12.5 g of Et_3N in 500 ml of CH_2Cl_2. The medium was stirred under argon pressure at room temperature for 4 days. Hydrochloride was then filtered off and the solvent removed *in vacuo* at 30 °C. The residue was washed three times with 100 ml of Et_2O so as to eliminate unreacted SPIRO (SPIRO is soluble in Et_2O whereas SPMCl is not). The insoluble residue in Et_2O was then washed three times with cold water (to eliminate residual traces of hydrochloride) and suitable extractions with CH_2Cl_2 leading finally to 9.5 g (43%) of SPMCl.

^{31}P NMR spectrum of SPMCl (101.27 MHz) is presented in Fig. 3. This 'ABC-like' spectrum essen-

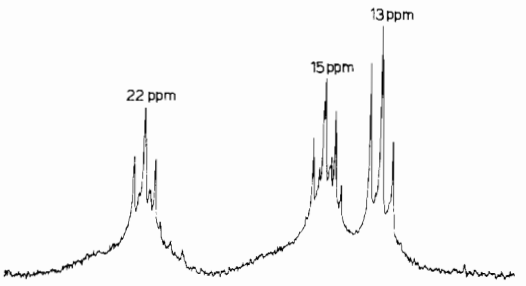

Fig. 3. ^{31}P NMR spectrum of SPMCl (101.27 MHz).

tially displays three false triplets which are actually split in doublets of doublets at 162.08 MHz. Such a set of three doublets of doublets is fully consistent with the assumed structure of Fig. 2 for SPMCl.

By comparison with spectra previously recorded in our laboratory, for some cousins of SPMCl [22], we may assign the three multiplets of Fig. 3, centered on 13, 15 and 22 ppm, to P(SPIRO) (A), P(SPIROBINO) (B) and $P(Cl_2)$ (C) atoms respectively. Coupling constants J_{AB}, J_{AC} and J_{BC} are equal to 45, 40 and 37 MHz.

Incidentally, high resolution NMR is a suitable tool for controlling elimination of the unreacted SPIRO starting material: the ^{31}P spectrum of SPIRO displays a triplet centered on 8 ppm which disappears when washed with Et_2O (see above).

To revert to the spectrum of Fig. 3, it is noticed that some suspicious sub-structures are revealed below each of the three multiplets we have just assigned. What is the origin of these sub-structures, thin layer chromatography, revealing one unique spot (actually broad, however) whatever the eluent? The answer was provided under peraziridinolysis of SPMCl as detailed below.

2 g of aziridine in 100 ml of THF were added dropwise in 1 h to 3.6 g of SPMCl in 200 ml of THF. The medium is stirred under argon pressure in an ice-bath. The reaction takes 24 h and is considered as completed when the IR stretching frequencies (540 and 580 cm^{-1}) of the P–Cl bonds in SPMCl have disappeared. Hydrochloride is then filtered off, the solvent is removed *in vacuo* at ambient temperature to give a residue which, upon recrystallizations from dry CCl_4, yields the final compound (I) (3 g, 80%).

Persubstitution of Cl atoms of SPMCl by Az groups is simple and quantitatively passes through in few hours, being controlled by high resolution ^{31}P NMR: multiplets of SPMCl centered on 13 and 15 ppm completely disappear after 24 h of reaction.

The ^{31}P NMR spectrum of (I) (101.27 MHz) is presented in Fig. 4. This spectrum displays two false triplets (actually doublets of doublets) centered on 18.9 and 20.7 ppm and a doublet of doublets centered on 38.8 ppm. Such an 'ABX-like' pattern may be assigned in a very simple way: the 38.8 ppm mass

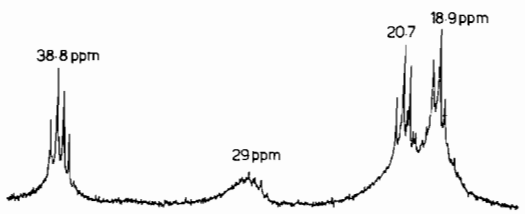

Fig. 4. ^{31}P NMR spectrum of (I) (101.27 MHz).

corresponds to the $P(Az_2)$ (X) atoms and the two others can be attributed to P(SPIROBINO) (A) and P(SPIRO) (B) atoms, respectively. Coupling constants J_{AB}, J_{AX} and J_{BX} are close together, 33, 34 and 36 MHz.

However, the spectrum of Fig. 4 also reveals the three expected multiplets on a broad band around 29 ppm and some unresolved sub-structures beneath the 18.9 and 20.7 ppm multiplets. The broad band around 29 ppm solves the riddle: it could be attributed unambiguously [21] to the spermidino homologue of (I) (Fig. 5) in which two SPIRO-$N_3P_3Az_2$[HN–

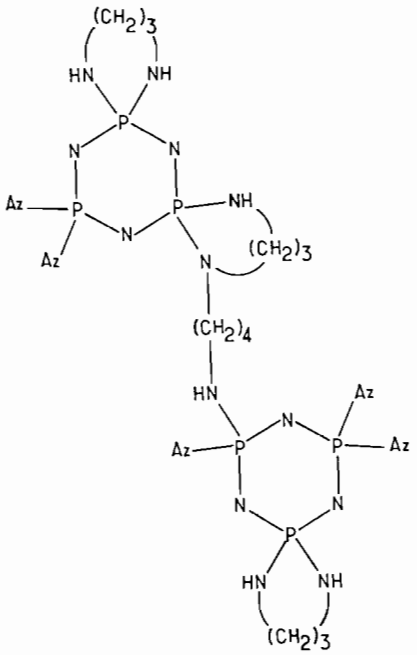

Fig. 5. The spermidino homologue of (I).

$(CH_2)_3$–NH] moieties are bridged through a spermidino entity in a SPIROBINO configuration [21, 23]. Such a spermidino impurity in (I) derives from the fact that the grade of the spermine used for the synthesis of SPMCl (FLUKA No. 85590) is only 96%, its main impurity being definitely spermidine.

Thus, peraziridinolysis of SPMCl reveals the nature of the impurity which is responsible of the doubtful sub-structures of Fig. 3 as mentioned above: this impurity is actually the spermidino homologue of SPMCl (Fig. 6) in which two SPIRO–$N_3P_3Cl_2$[HN–$(CH_2)_3$–NH] moieties are bridged through a spermidino entity in a SPIROBINO configuration [21, 23]. This conclusion is definitely supported by recording a ^{31}P NMR spectrum of SPMCl at 162.08 MHz, ABC-type spectra of SPMCl and of its spermidino homologue being then split

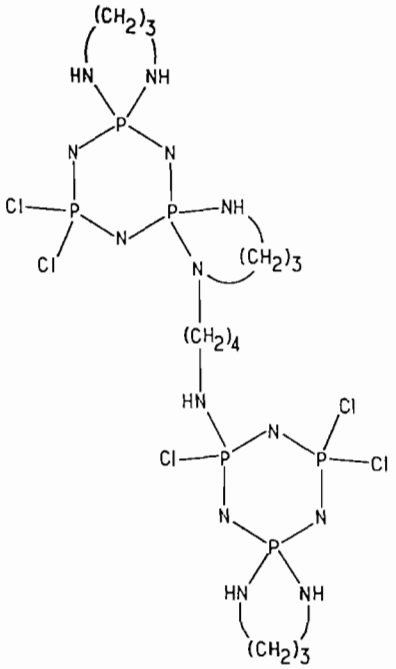

Fig. 6. The spermidino homologue of SPMCl.

enough to make their prime attribution unambiguous.

However, the final spermino product (I) contains a small amount of its spermidino cousin and all techniques for eliminating this spermidino impurity have failed so far. The only way to avoid this dilemma would be to use pure spermine as a prime material but, so far, nobody seems able to supply spermine without spermidine as a side-product. Consequently, any prepared spermino derivative must be expected to be contaminated *de facto* with small but uncontrollable amounts of its spermidino cousin. The synthesis reported here emphasizes clearly that the obtention of pure spermino derivatives constitutes, in 1985, a challenge to both the chemist and the biochemist.

Despite the questionable grade of purity of (I) as noted, we decided to go further and make a spot test of antitumor activity *in vivo*.

Antitumor Activity of (I)

Antitumor tests were performed at the Mario Negri Institute (Milan, Italy) by Drs. Federico Spreafico and Stefania Filippeschi within the framework of the activities of the EORTC Screening and Pharmacology Group.

The P388 murine leukaemia was chosen as the tumor model system. 10^6 cells were transplanted intraperitoneally (i.p.) on day 0 in compatible $CD2F_1$ male mice (20 ± 2 g), the drug treatment being initiated 24 h later. The drug was dissolved in sterile saline (owing to its high water solubility, larger than 20 g l^{-1}) and administrated i.p., ten animals per group being used. Results presented are representative of at least 3 experiments so performed. The percentage increase in median lifespan over untreated controls (T/C%) was calculated as an evaluation criterion of effectiveness [24]. T/C% is significant for antitumor activity only when higher than 125%.

Very preliminary data are reported in Table I, no attempts to identify optimal treatment schedules being made.

TABLE I. Effect of (I) on P388[a] Leukaemia in $CD2F_1$ Mice.

Treatment schedule dose (mg/kg/i.p.)	T/C %
200 × d 1	150
100 × d 1	135
50 × d 1	140
25 × d 1–9	140
12.5 × d 1–9	140
6.25 × d 1–9	120

[a] 10^6 cells were implanted i.p. on day 0.

Nevertheless, T/C% values higher than 125 are observed for 5 protocols over 6, both through single injections (200, 100 and 50 mg/kg on day 1) and within chronical polyinjection schedules (50, 25 and 12.5 mg/kg every day for 9 days). Compound (I) then displayed a poor but significant antitumor activity.

Of course, there could be objections to the real significance of this effectiveness owing to the defective purity of (I). However, systematic investigations of antitumor activity for spermino and spermidino derivatives of cyclophosphazenes now in progress in our laboratory [17] prove that the former are commonly more active than the latter. Thus, the real effectiveness of pure (I) could be reasonably expected to be still higher than the sample studied here.

In conclusion, we can fairly confidently suggest that compound (I) displays significant antitumor effectiveness, on at least P388 leukaemia. Thus, the covalent binding of non-effective diaziridinocyclophosphazenic principles *per se* to two 1,3-diaminopropano groups (in SPIRO configuration) and one spermino group (in a DISPIROBINO configuration) as tumor finders lead to an effective antitumor agent. In other words, the linkage of an ineffective drug to natural polyamines as tumor finders reveals its significant effectiveness. We may reasonably assume that this result is due to an increase in the bio-availability of the drug through such a vectorisation. Moreover, the presence of two pairs of Az ligands on the same N_3P_3 ring no longer seems dogmatic for antitumor effectiveness.

Acknowledgements

We are greatly indebted to Dr. B. Monsarrat for the collection of mass spectra and to Dr. G. Commenges for high resolution NMR data. Part of this work was performed within the framework of the EORTC Screening and Pharmacology Group activities. The authors are grateful to the French CNRS-ANVAR Research Fund for its financial support for the biological part of this work and the Toulouse Football Club for its psychological support to one of us (J.L.S.).

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