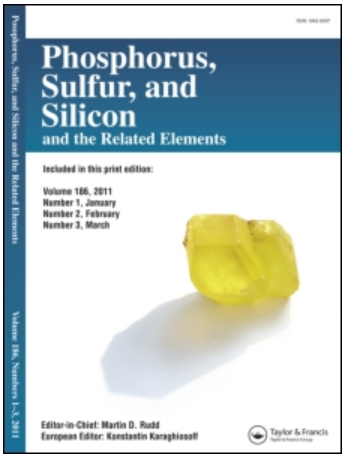

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Anticancer and Immuno-Modulating Properties of Cyclophosphazenes

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ANTICANCER AND IMMUNO-MODULATING PROPERTIES OF CYCLOPHOSPHAZENES.

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Abstract Clinical uses of aziridinocyclophosphazenes ("nude" drugs) as anticancer immuno-modulating drugs are severely limited by hematological toxicity.Benefits from vectorization through covalent linkage to biogenic polyamines as tumor finders are evidenced and further improvements through monoclonal antibodies are envisaged.

INTRODUCTION

Actually, great strides of Science were more often made from wrong hypotheses and through unsound reasonings. Scientists are then de facto cyclothymic, moving as yo-yos from over-excitements when ideas seem to loom up to hyper-frustrations when Nature says no. It is no wonder that Researchers are doomed to live as on roller coasters, now sad now gay, with hopes and disillusions as everyday bread and heart-attacks in the end. However that may be, Research Work stays the most fantastic job since one is labored under gold-digger (or kamikaze) spirit.

Events that stood up as milestones the story of cyclophosphazenes as antitumor immuno-modulating drugs did not escape the rule and the present contribution intends relating both infernal and paradisiac times we lived in our Laboratory for the last ten years.

OBSTACLE COURSE N° 1 (1975-1982) : THE "NUDE" DRUGS[1].

In 1975, it was generally assumed by people in charge of anticancer drugs molecular design that the main target to be considered in malignant cells was DNA. Indeed, all drugs used in clinics at that time were classified either as dialkylating or as intercalating antimitotics, except of course alcaloids from *vinca rosea* , i.e. vincristine, vinblastine and congeners. In other words, any molecule capable of reaching DNA of tumor cells and to link it through double covalent binding (dialkylation) or through suitable hydrogen bonding with polar heads of A, T, G, C amino-acids (intercalation) could be expected to be anticancer agents. Of course, intercalation implies molecules flattened enough to creep between DNA plates and dialkylation presupposes the existence in the molecules of two labile alkylating groups in *cis* position susceptible to attack amino-acids, as plugs in a way. Incidentally, it was commonly claimed that the most convenient distance between the two leaving groups for an optimal dialkylation was 3.3 Å, according to the molecular geometry of *cis-Platinum* , $(NH_3)_2PtCl_2$, where d(Cl...Cl) is exactly equal to this "magic number".

In 1975, we were dealing with "keys" design within the field of Coordination Chemistry with the aim of conceiving molecules which would "open the lock" of problems related to catalysis and polymers. Actually, we were involved inconsciously in "key-lock", "yin-yang", "drug-receptor" approach, even if at that time we were not yet obsessed by drugs design. Suddenly, on June 21, 1975, when attending a Conference on "up-to date improvements in Cancer Chemotherapy", we heard that the "lock" of Cancers was DNA with its "magic" keyhole, 3.3 Å. Actually, we were working from more than five years within the international pool devoted to Cyclophosphazenes with R.A. SHAW, M. WOODS, T.S. CAMERON, S.S. KRISHNAMURTHY and many others and the basic molecule of the series, discovered in 1834 by ROSE and LIEBIG, is the hexachlorocyclotriphosphazene, $N_3P_3Cl_6$ in which three pairs of Cl atoms do exist in geminal position with ... d(Cl...Cl) = 3.3 Å.

Imagine what happened ? Leaving computers (without any remorse) for mice, we engaged in vivo tests with $N_3P_3Cl_6$ and cousins (i.e. aziridino derivatives , good likenesses of Thiotepa, TEM and Dipin, dialkylating drugs widely used in

clinics at that time) on murine (i.e. in mice) tumors (Leukemias P388 and L1210, melanoma B16, Sarcoma 180, Lewis Lung Carcinoma) and six months later we had observed our first very significant ILS (Increases in Life Span) on every tumor, with single or short QnD polyinjection protocols.

First over-excitement, beginning of the obstacle course n° 1. We synthesized and tested in vivo several aziridino-chloro-cyclophosphazenes, $N_xP_xAz_mCl_{x-m}$, with x = 3 (trimers), 4 (tetramers) and 5 (pentamers) and m = 1 to 6, together with some cyclophosphazenes-like in which P atoms had been replaced by S ones. In 1979, the situation could be summarized as follows. Any drug with x = 3 was in every respect more effective than the ones with x = 4 or 5. Moreover, m = 4 or more gave very active principles when m = 2 or less yielded uneffective molecules. Then, x = 3 and $m \geq 4$ seemed to be the market gap where new drugs could be designed for future clinical applications.

And that was how we investigated parallely $N_3P_3Az_6$ (MYKO 63), gem-$N_3P_3Az_4Cl_2$ (MYCLAZ) and $N_3P_2SOAz_5$ (SOAZ) and patented the latter (with J.C. Van de Crampel and A.A. Van der Huizen) in 1979. The whole molecules were active on any murine tumor they were tested on but their biological behaviour was vitiated by a dose-dependent hematological toxicity (mainly leucopenia and thrombopenia) which stopped their development for clinical uses, even if they did not display either nephro-, hepato- or cardio-toxicity , or allopecia or necrosis when injected by intravenous routes.

Such a penalizing side-toxicity was even observed more recently by VAN DE GRAMPEL et al. with trans-$N_3P_3(NHMe)_4Az_2$ (AZP) and trans-$N_4P_4(NHMe)_6Az_2$ (AZM)[2] and we may actually conclude from that period devoted to what we call "the nude drugs" that one cannot recommand the development of such aziridinocyclophosphazenes for future clinical purposes.

These misfortunes (hyper-frustrations n° 1 to 5, one per drug) are basically due to the very poor therapeutic index, T.I., of the nude drugs. T.I., i.e. the ratio of the maximal non-lethal dose LD_0 to the minimal active dose in single injection D_{min}, never exceeds indeed 4 to 5, making single injection protocols with D_{min} already toxic (D_{min} being too close to the toxic level) and prohibiting de facto polyinjection protocols which lead to cumulative toxicity.

Such is the desperate cul-de-sac where "nude" drugs are trapped (no

reasonable hope of clinical applications, even if their hematological toxicity is actually and honnestly comparable to the ones of other drugs which are commonly and daily used in clinics), despite of remarkable effectivenesses in vivo on murine tumors. What could be done for cancelling - or at least attenuating - this specific side-toxicity, remembering that it is strictly dose-dependent ?

Several tracks could be attempted, according to the tremendous multidisciplinary efforts which have been made during the last decade for enhancing the selectivity (and, consequently, for decreasing therapeutic doses) of anticancer drugs, essentially through covalent bindings either to monoclonal antibodies (immunoglobulins) or to natural polyamines (mainly putrescine, cadaverine, spermidine and spermine), antibodies and biogenic polyamines playing the role of tumor finders and, in few cases, of homing heads. These approaches were diversely successful but they are definitely the only way to escape from the uncomfortable dilemmas allopathy in cancer chemotherapy nowadays comes up against.

We selected in a first step polyamines as tumor finders, according to the outstanding works from Carl PORTER et al.[3], and the next paragraph will summarize the Chemistry, the Architecture and the benefits of the covalent binding of "nude drugs" to biogenic polyamines.

OBSTACLE COURSE N°2 (1982-1987): THE VECTORIZED POLYAMINES-LINKED CYCLOPHOSPHAZENIC DRUGS[4].

Reactions of natural polyamines with $N_3P_3Cl_6$ give unique products the structure of which depends on the nature of the polyamine, on the stoichiometry and on the way of addition (which is added to which). Indeed, when two moles of the diamine are added dropwise to one mole of $N_3P_3Cl_6$ in aprotic solvents such as acetonitrile, diethylether, dichloromethane and/or THF, diamines are grafted on $N_3P_3Cl_6$ stereoselectively and stereospecifically either as a SPIRO loop (1,3-diaminopropane and putrescine) or as a BINO bridge (cadaverine and higher homologs) when triamines (such as spermidine) and tetraamines (such as spermine) link naturally $N_3P_3Cl_6$ in the so-called SPIRO-BINO and DISPIRO-BINO configurations. By using the same way of reaction and just modifying the

stoichiometry, one gets DISPIRO, TRISPIRO (symmetrical or merged), non gem-DIBINO and non gem-TRIBINO (BARRELANES) derivatives neat. Thus, SPIRO, BINO, SPIRO-BINO and DISPIRO-BINO configurations may be considered as the innate ones which currently occur upon grafting of biogenic polyamines on $N_3P_3Cl_6$. In other words, the BINO and SPIRO configurations, together with the serendipitous ANSA[5-7] one [obtained from the bis-(2-aminoethyl)-ether], constitute the three basic bricks of the B.A.S.I.C. (Bino Ansa Spiro In Cyclophosphazenes) chemical game which allows to link on demand several SPIRO loops, ANSA arches and/or BINO bridges on a cyclophosphazenic species.

In other respects, the use of protic solvents such as chloroform (with the same way of addition) favours DANGLING configurations in which the linked polyamine keeps one amino function free at least. We recently reported on some BIDANGLING and SPIRO-BIDANGLING forced new configurations[8] we prepared from spermidine and spermine and on the DANGLING kinetic product revealed upon reaction of the bis-(2-aminoethyl)-ether on $N_3P_3Cl_6$ in chloroform.

Conversely, when one mole of $N_3P_3Cl_6$ is added dropwise to two moles of polyamine in high dilution conditions with chloroform as the solvent, one gets M.A.G.I.C. (Merged Architectures Generation In Cyclophosphazenes) systems such as the SPIRANSA[9], SPIRANSA-SPIRO and DISPIRANSA structures. These appelations are actually contractions of the ones of B.A.S.I.C. bricks to point out that the SPIRO loops and the ANSA arches in M.A.G.I.C. systems have one (P-N) bond in common at least, forbidding then to call them SPIRO-ANSA, SPIRO-ANSA-SPIRO and DISPIRO-ANSA. Indeed, B.A.S.I.C. systems are built *stricto sensu* from independent bricks lying on different phosphorus atoms of the ring without any overlap (atom or bond). Incidentally, SPIRO-BINO and DISPIRO-BINO appellations of innate configurations from spermidine and spermine we evoked above are basically inadequate, SPIRO loops and BINO bridges having indeed nitrogen atoms in common. Thus, they have to be replaced by SPIRBINO and DISPIRBINO, appellations which are quite consistent together with B.A.S.I.C. and M.A.G.I.C. rules.

Up to now, more than thirty various B.A.S.I.C. and M.A.G.I.C. systems were prepared in our laboratory with the aim of producing more and more

selective antitumorals. Nucleophilic substitution of the whole chlorinated precursors we just described yields indeed to vectorized polyamines-linked drugs both the water solubility and lipophilicity of which drastically depend on the configuration of the polyamine. For example, SPIRO loop-bearing drugs are soluble in physiological serum at 2 kg l^{-1} with relatively poor lipophilicity when the solubility of BINO bridge-bearing drugs is less than 2 g l^{-1} with a correlatively high lipophilicity. In other words, if a very high water solubility is an advantage for drugs which have to be injected through intravenous routes the concomitant poor lipophilicity may be a disavantage, mainly when bioreceptors of drugs are phospholipidic sites.

Chronologically, the first vectorized drugs we prepared and tested were the peraziridino derivatives of the SPIRO chlorinated precursors from 1,3-diaminopropane and putrescine[10]. These drugs were labelled as DIAM3 and DIAM4, 3 and 4 being then the number of methylenic groups into the SPIRO loop. Acute toxicity studies in vivo immediately evidenced the first benefit from linking a biogenic diamine to the "nude" drug: The therapeutic index T.I. of DIAM3 and DIAM4 is raised up indeed to 10 for the former and 15 for the latter, versus 4 to 5 for "nude" drugs, remember. Then, single injection protocols can be performed on a large scale of non-toxic or poorly toxic doses and, above all, chronical polyinjection schedules (with small daily doses) can be achieved on very long periods (several weeks or even months) without emergence of significant cumulative toxicity. It will be shown below how such posologies allow to treat cancers and also - what is more important yet perhaps - auto-immune diseases which are basically much more slow-evoluting than cancers and then require very long chronical treatments.

In a second step, we synthesized and tested the peraziridino drug of the DISPIRBINO innate chlorinated precursor from spermine. This new drug was labelled as SPM[11] and once more its therapeutic index appeared to be very high, i.e. about 30. However, we did not develop it owing to the too low intrinsic toxicity of SPM (LD_0 = 1500 mg/Kg) which makes the corresponding D_{min} equal to 50 mg/Kg, versus 5 mg/Kg for DIAM3 and DIAM4. In other words, for economical and technical considerations, we decided to shelve temporarily SPM and to develop DIAMs.

World patents covering DIAM3, DIAM4 and SPM were purchased by PIERRE FABRE MEDICAMENT as executive licensee and this Company is currently preparing the pharmacological and toxicological file for DFF agreement. Clinical phases I and II are now running and one may understand that publication of such results does not come within the scope of my duties and will be made further by clinicians themselves.

Anyhow, now is the time to state the facts which make us confident in these new vectorized drugs (over-excitement n° 2). First of all, the effectiveness in vivo of these drugs was demonstrated not only on murine tumors as previously (almost any molecule indeed may be found active on such "models" and actually inactive on human tumors) but actually on human cancers grafted in nude mice , which is much closer to Reality. The prime hopes we got in this way came from experiments which were performed by Dr. F. DARCEL and her I.N.S.E.R.M. team at Rennes Hospital (France): Three human glioblastomas (brain tumors, namely Aussant, Coadalen and the standard U 251 from Uppsala Hospital) were grafted on nude mice and cured with both DIAM3 and DIAM4 by using a chronical protocol (10 mg/Kg daily 5 days a week, no treatment on week-end, and start again) for 8 weeks. Control mice died on day 37 with tumors the volume of which is about 7 grams (for a mean body weight of 23 grams). Treated mice on day 100 are bearing a barleycorn-like cystic residue with a very thick peel and few residual polyploidic cells with very poor capability to mitosis. More important yet perhaps : only few renewals of the tumor-growing were observed, on day 200, when stopping the treatment on day 100. Incidentally, hematological toxicity with DIAM4 after 20 weeks (i.e. 5 months) of treatment in sequence non stop stays grade 1 (according to the W.H.O. scale), that is definitely acceptable. Such a cheerful result shows clearly that anticancer drugs with high therapeutic indexes have to be used in chronical schedules with minimal doses daily and surely not, as done too often, in single overdose shots. In other words, contrarily to what is commonly done in cancer chemotherapy, allopathy with antitumorals must be achieved according to very smooth protocols on long periods and not through few "from time to time" toxic jabs. I may say that this opinion is more and more shared by clinicians provided that drugs they use have large T.I..

A second hope comes from the remarkable activity of DIAM3 and DIAM4 on

Systemic Lupus Erythematosus (SLE), a well-known auto-immune incurable disease. The story of this discovery is worth relating to prove, if necessary, advantages of multidisciplinarity with eavesdropping people. Lecturing in Paris on June 1983, I mentioned that lymphocytes B were the most affected amongst the whole white cells by DIAMs toxicity. When leaving the lecture room, I was accosted by Dr. G. FOURNIE, from I.N.S.E.R.M. Unit n° 269 (Toulouse Hospital , France) who told me this : "Are you conscious that your DIAMs are probably active on SLE?". I did not know at all at that time what was SLE ! I gave him two days later 100 mg of DIAM4, let me say "just for pleasure", and I waited.

Two months later, the effectiveness of DIAM4 on SLE-genetically injured mice (I insist, on naturally diseased mice and not on some induced "model" SLE systems) was clearly demonstrated. Let me sum up here what is SLE. A bacterial endotoxin (such as a lipopolysaccharid, LPS) which normally would circulate in the body, suddenly grips - for unknown reasons - some receptors of endothelial (in blood vessels) walls.The presence of this intruder launches attacks of lymphocytes B (which are polyclonally stimulated) and of suitable immunoglobulins (the rate of which increases sharply for some of them and decreases sharply for their complements) but lymphocytes and IGs do not succeed to unbind LPS. Now, the human body comprises too much IGs which are inducing pathological effects in it and it tries to eliminate these cumbersome IGs as commonly, that is through urinary routes. But these IGs in large excess give immune complexes in kidneys which "clog the filter", leading to severe glomerulonephritis. Patients have then to resort to hemodialysis and/or plasmapheresis, under corticoids medications, till a delayed but inescapable end.

The present situation (late March 1988) is as follows. Chronical protocols with 10 mg/Kg daily from Monday to Friday, stop on the week-end and start again (identical to the ones used by Dr. F. DARCEL in Rennes for glioblastomas) cure genetically-injured mice both in preventive and in curative treatments, that is when initiating its either before emergences of objective pathological signs or when animals are starting to die. Chronical posologies take from 6 to 10 weeks to get animals cleaned out rather perfectly. No renewal of SLE disease was ever observed on day 15O with animals where treatment was stopped on day 7O. This is extremely surprising, owing to the genetic character of this disease , and would

mean that genes responsible for such an auto-immune disease would be inhibited to some extent upon DIAMs intervening.

The present contribution being devoted to Chemists, I don't wish to present here more biological and medical things. However, let me say that fundamentalists we are are confronted to something great - we have of course to elucidate why - which is "Aziridinocyclotriphosphazenes, "nude" or "vectorized", are active both on certain sorts of human cancers and on human auto-immune diseases". When considering medical courses, these two kinds of diseases are generally considered as independent and activities of DIAMs we just mentioned prove that there is something in common between the two devils. But where ? Would there exist a common trunk somewhere on which the drug would be active ? We urge solemnly any link in the chain going from ideas (Chemists) to medical acts (Clinicians) to join us without any a priori for solving a problem which overdoes the limited field of cyclophosphazenes as drugs.

Coming back to effectiveness of DIAM4 on SLE, we may say that the drug displays significant activity on every step of the disease (i) inhibiting polyclonal stimulation of lymphocytes B (step 1), (ii) modulating activation (or desactivation) of IGs and complements (allowing to qualify DIAM4 as immuno-modulator and not as immuno-suppressor)(step 2), (iii) and making deposits of immune complexes in kidneys regressing when the disease reaches step 3 level. In other words, vectorized cyclophosphazenes appear as a prime and really new hope for clinical treatments of auto-immune diseases such as SLE of course but also such as Rhumatoid polyarthritis, multiple sclerosis and non-insulino-depending diabetes. Unfortunately, there does not exist, to our knowledge at least, any feasible model in animals which could allow approaching eventual therapies of the latter for humans, as it is the case for SLE.

Keep quiet, ladies and gentlemen. The whole things presented here will have to be confirmed or invalidated by clinicians within the very next few months. Again, we shall live over-excitements or hyper-frustations but we know from the beginning that it is our lot. However, in order trying to escape from an eventual negative verdict , we took the opportunity to move to the most "royal" way of targeting, that is to attempt at the production of more selective antitumorals yet through covalent binding of "nude" drugs and/or of vectorized drugs to monoclonal

antibodies. This new approach just started on the last week with Dr. P. PELLEN (from Rennes Hospital once more) and I hope to be able to present preliminary results in Amherst next August, hoping feverishly that "it will not be a lot of fuss for nothing".

Another approach for designing vectorized drugs which would be more compatible yet with human harmony consists in introducing oxygen atoms into the SPIRO loops, the ANSA arches and the BINO bridges, according to CRAM, PEDERSEN and LEHN works. The biological interest of oxodiamines is now indeed well-documented, crown-ethers and cryptands being widely used within the fields (i) of activation and stabilization of anions and cations of biological interest (ii) of selective recognition of cations (iii) of co-receptor cryptands and (iv) of cooperativity.

By our own, we were urged naturally in 1986 to look at the synthesis of BARRELANES (i.e. non-gem TRIBINO derivatives) which would be more soluble in organic polar solvents (and, eventually, in water) than the purely hydrocarbon species. A correspondence with Jean-Marie LEHN and Fernando MONTANARI, dated on March 21, 1986, urged us to replace normal biogenic diamines by isologous oxodiamines for the preparation of new macrocyclic vectorized cyclophosphazenes. These works are now running nicely in our Laboratory and we just may say that several sorts of cyclophosphazenic exotic species are now available in large quantities (about 5 grams each), with H_2N-$(CH_2)_3$-O-$(CH_2)_4$-O-$(CH_2)_3$-NH_2 (coded as 30403)[12], as the ligand. Similar derivatives are now synthesized from 20202 (Jeffamine ED 148, TEXACO gift) and from 30203 (BASF gift) . X-Ray structures of these cyclophosphazenic species are in progress in our Laboratory.

We started this lecture in insisting on the wrong hypotheses and the unsound reasonings which preside over the making of great strides of Science and we suggested that the story of cyclophosphazenes as anticancer immuno-modulating drugs did not escape this rule. Then, it is now the time to prove the well -founded background of this assertion.

WRONG HYPOTHESES AND UNSOUND REASONINGS IN DESIGNING CYCLOPHOSPHAZENES AS DRUGS.

Remember 1975 when we got the idea that $N_3P_3Cl_6$ and aziridino derivatives could act as antitumor agents through a dialkylation of vicious DNA. Whilst we were successfully demonstrating their in vivo effectiveness, investigations on mutagenicity (as a consequence of interactions with DNA) were performed (Ames tests with and without microsomes) together with an in vitro study of interactions with purified DNA (Scatchard technique). And results came : Cyclophosphazenes are not at all mutagenic, at non-toxic levels at least and they poorly interact in vitro with DNA, even after incubation times larger than one week at 37 °C. It must be emphasized that classical dialkylating agents link DNA in same conditions in less than ten minutes and give significant numbers of revertants (mutants) in Ames tests. Then, effectiveness of cyclophosphazenes we could attribute to a dialkylating effect appeared to be definitely due to something else. First wrong hypothesis.

The non-mutagenicity of "nude" drugs was confirmed in 1981 when studying the way of action of SOAZ with streptococcus pneumoniae and L 1210 cells as the biological targets. Streptococcus bacteria is mutant-sensitive to practically any chemical but it is not to "nude" drugs. Then, this experiment , quite surprising for biochemists, prove if necessary that the kind of drugs we are dealing with do not exhibit any mutagenicity. Moreover, the same study showed that SOAZ does not penetrate the cell at all, dialysis of incubated cells releasing the totality of the drug free. More recently, PORTER investigated for us an eventual competition of DIAM4 (where putrescine is linked to the "nude" drug in a SPIRO configuration) with free tritiated putrescine with respect to the uptake by malignant cells in vitro. No competition was observed, proving that even the vectorized drugs do not penetrate the cell. All these observations , which were so upsetting for tough nuts whose Credo was "cyclophosphazenes are dialkykating drugs, full stop", made us happy. That is so agreeable to laugh at oneself! The only think to be considered at that time was "drugs are effective", nothing more. So much the more that it was demonstrated few months later that nude drugs like SOAZ do not link cell membrane, just collapsing the trans-membranar electric potential, $\Delta\Psi^*$, and drammatically modifying the transport through the membrane of $\Delta\Psi^*$-dependent

amino-acids. Then, malignant cells would be killed by a sort of amino-acids bulimia, which is supported by the huge increase of the size of incubated malignant cells as observed by electron microscopy.

However, the latter observation was made in vitro and people dealing with cyclophosphazenes know that there is no clear relationship between in vitro and in vivo data. For example, in vitro measurements of the ID_{50} inhibiting dose on L 1210 growing cells would have inclined to screen $N_3P_3Cl_6$ as the best antitumor agent through the whole series of candidates (the largest ID_{50} of all) when we know now that it is amongst the poorest drugs in the panel. Incidentally, the discrepancy between in vitro and in vivo tests suggests , according to what happens for other types of anticancer drugs, that either "nude" or vectorized cyclophosphazenic drugs would be conveyed in vivo by some protein-carrier which would protect the drug from a too large and too fast metabolization. This is supported by the fact that "nude" and vectorized drugs are excreted in vivo for a large amount "like they are", i.e. without any metabolization, the remainder being excreted (through urins and feces) as slightly modified molecular structures versus the original one.

Anyhow, from the whole previous results, we get the feeling that biogenic polyamines do not play probably the role of tumor finders the design of vectorized drugs was based on. Steric hindrances due to covalent linkages of these polyamines in innate and/or forced configurations presumably make our gundogs loosing their smell to some extent. This is probably the second wrong hypothesis we worked from for the recent past but high therapeutic indexes we got do remain and this is, let me say, essential for future. More than 85 % of drugs which are commonly used successfully in clinics have definitely unknown modes of action, clinicians say, and however they save people.

The real knowledge of anticancer immuno-modulating cyclophosphazenes mode of action will take time but tumor-bearing and auto-immune diseases-suffering patients cannot wait longer and clinicians, who are daily confronted to incurable patients know, and they are alone to know, what they can or cannot do, in agreement of course with patients and families.

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