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## RECOMMANDATIONS FOR TOXICOLOGICAL EVALUATION OF NANOPARTICLE MEDICINAL PRODUCTS

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## **Recommendations for toxicological evaluation of nanoparticule medicinal products**

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***(First edition available upon request)***

### **A. INTRODUCTION**

Nanoparticles are defined as particles at dimensions between approximately 1 to 100 nanometres in at least one dimension. They have considerable general industrial applications and although only a few nanoparticle medicinal products are available at the present time, a spectacular development of these products can be expected over the years to come. In the short and medium term, the main use of nanoparticle medicinal products (NMP) is for vectorization of active principles, corresponding to several products already marketed.

Three types of vectors are distinguished at the present time:

- First generation vectors: nanospheres and nanocapsules (the best known and most accessible),
- Second generation vectors: nanoparticles coated with hydrophilic polymers such as polyethylene glycol (PEG), pegylated nanoparticles
- Third generation vectors, still under development, combining a biodegradable core and a polymer envelope (PEG) with a membrane recognition ligand.

Very generally, nanoparticle medicinal products are colloidal systems that can be classified into three classes:

- 1) biodegradable nanoparticles,
- 2) soluble nanoparticles,
- 3) insoluble or slowly soluble nanoparticles.

Biodegradability or solubility is an important property, determining elimination of nanoparticles introduced into the body. Other parameters than the conventional parameters (mass, mass per unit volume) must also be taken into account in order to relate their effects to exposure: for example, the relative size of the particles, their active surface area (total external and possibly internal surface area of exchange), number of particles per unit volume, etc. These parameters largely determine uptake, distribution, and elimination of NMPs. It is also absolutely essential to take into account the formation of nanoparticle agglomerates, that subsequently form aggregates the characteristics and potential risks of which being very different from those of the initial nanoparticles. These agglomerates and aggregates also form during storage in a liquid medium. In a biological medium (blood, plasma), this formation may be facilitated, though the opposite phenomenon has also been reported, i.e. the regeneration of initial nanoparticles. This means that in practice, the behaviour of the nanoparticle form must be known, in particular concerning its structure and property changes in excipients or culture media used during tests (particularly in the presence and absence of serum, specific proteins, etc.)

In addition to the specific toxicity of the vectorized active principle, the structure in which it is contained could also considerably modify this toxicity. Consequently, it would often be preferable to consider the NMP as a distinct entity that needs to be evaluated as a largely new "total" drug substance. The nanoparticle form can also induce specific risks (formation of agglomerates), transport impurities by adsorption, generate toxic substances by degradation or dissolution of the constituents of the NMP, cross physiological barriers (blood-brain, foetoplacental, cell and nuclear membranes, etc.). This illustrates the magnitude of the task of toxicological evaluation of NMPs, especially

since part of this field is only very poorly documented, on the one hand, and no reference material is available to evaluate nanoparticles, on the other hand. Under these conditions, to ensure better efficacy (especially for screening) and for ethical reasons (unjustified and extensive use of laboratory animals), the use of validated *in vitro* methods must be strongly encouraged. These methods must ensure relevant evaluation of genotoxicity, cytotoxicity, free radical formation, biopersistence, phagocytic capacity, etc.

It should be stressed that although vectorization of active principles is probably the major potential use of nanoparticles in medicine, other very valuable applications must be considered: tissue engineering and diagnostic tests, for example. Nanoparticle formulations are also widely used in cosmetology (sunscreens) and are a subject of controversy in some countries. Finally, it is also somewhat surprising to find so many scientific publications concerning the occupational and environmental risks of nanotechnologies and nanoparticles, compared to the limited number of documents concerning NMPs.

## **B. OBJECTIVE**

The recommendations presented in this position paper reflect the opinion of the Afssaps Working Group on Non-Clinical innovation. They are therefore opened to reflection and discussion, especially as some proposals are essentially pragmatic, sometimes even empirical. In view of the potentially extensive nature of the field of NMPs, the Working Group has decided to limit its investigations to three sectors already developed or under development:

- Use of NMPs in medical imaging (MRI and ultrasound),
- Vectorization of drug substances (anticancer drugs, antibiotics, antifungal agents, etc.), by introduction of NMPs into the body,
- Use of NMPs by topical routes (skin, lung, eye, etc.) in order to obtain systemic exposure or a local effect.

Recommendations for the toxicological evaluation of NMPs will be formulated in the usual order of other guidelines. The Task Force emphasizes that, in view of the wide range of structures, physicochemical and biological properties, therapeutic uses, etc., case-by-case assessment of the most relevant study programme for a given NMP will always be essential.

## **C. RECOMMENDATIONS**

As indicated above, general scientific and/or regulatory data related to the toxicological evaluation of NMPs are currently lacking. However, we can refer to the European Commission (Health and Consumer Protection) document prepared by the SCENIHR (Scientific Committee on Emerging and Newly-Identified Health Risks) entitled "Opinion on the Appropriateness of the Risk Assessment Methodology in Accordance with the Technical Guidance Documents for New and Existing Substances for Assessing the Risks of Nanomaterials" and approved by public consultation on June 2007. Although not specifically dealing with NMPs, this document contains a number of considerations that can apply to NMPs. Also note that the "conventional" toxicological approach proposed by current guidelines for medicinal products in general (ICH, FDA, EMEA) has been accepted up until now for approved NMPs or NMPs currently under evaluation by health authorities. However, some criticisms have been raised concerning the currently available methods of experimental

evaluation that are considered to not adequately assess the properties of nanoparticle products. The most frequent criticisms essentially concern pharmacokinetic and toxicokinetic studies, which are considered to not realistically take into account the specificities related to the nanoparticle structure. The relevance of *in vitro* tests has also been questioned, as the sedimentation rate and diffusion capacity of nanoparticles must

modify the conditions of exposure (dose-duration) of the models used (for example, genotoxicity tests). Finally, the lack of data on long-term effects is often emphasized. Consequently, like certain consumer groups in the USA, we may need to recommend the development of completely new regulations based on "adapted" safety assessment tests for nanomaterials, including NMPs. This maximalist proposal is totally idealistic and scientifically unjustified according to the very great majority of the scientific community. How many years for development and validation would be necessary to achieve such a result? This major revision also does not appear to be justified by the available scientific data.

Some manufacturers consulted and most of the task force also consider that toxicological evaluation of NMPs should not be appreciably different from "conventional" evaluation, but with certain specific adaptations (inappropriate nature of repeated-dose studies for NMP used as a single dose in man, such as in medical imaging). The plan adopted for elaboration of these recommendations is based on this approach, i.e. adapt the safety assessment strategy, when necessary, without modifying the basic principles.

## **1. Pharmacokinetic studies**

Evaluation of absorption, distribution, metabolism and excretion (ADME) of nanoparticles and of their degradation or solubilization products is essential and must be performed prior to safety studies. The pharmacokinetic properties of nanoparticles are very different from those of conventional molecules but are nevertheless studied in similar ways. Four factors essentially determine the pharmacokinetics of nanoparticles: route of administration, particle size, nature of coating polymers and animal species. Biodistribution study methods should focus, on the one hand, on the material comprising the NMPs and, on the other hand on the associated active principle. For this latter, conventional analytical methods shall be used. For nanoparticles, an adapted labelling method will need to be used, ensuring that this labelling is not likely to modify the physicochemical properties of the nanoparticles and hence their biodistribution. It will also be necessary to ensure that this marker is not "prematurely" released from the nanoparticle, leading to potential confusion between monitoring of the released marker and that remaining bound to the nanoparticles. One means of proceeding for these studies would be to perform a double labelling, ensuring that both markers remain associated throughout the biodistribution monitoring process. The methods used are conventional: fluorescent or radioactive labelling. Scintigraphic, PET or fluorescence imaging techniques do not provide quantitative results, but are nevertheless suited to the study of the biodistribution of NMPs administered by parenteral, pulmonary or even enteral routes, along with to the determination of sequestration sites and translocation phenomena. For PET, radioisotopes with a relatively long half-life, such as  $^{64}\text{Cu}$  (T<sub>1/2</sub> = 12.7h) and  $^{89}\text{Zr}$  (T<sub>1/2</sub> = 78.4h) are particularly well-suited to the study of the later stages of biodistribution and translocation, over periods ranging from 36h to 9 days, when stable *in vivo* labelling is possible via a functionalised constituent of NMPs.

NMPs are frequently administered by parenteral route (IV, occasionally SC or IM), but also by specific local routes (lungs, skin, eyes). When administered by parenteral route, they are recognized by the reticuloendothelial system and phagocytosed by macrophages (liver, spleen, lymph nodes, bone marrow, lungs, etc.). This property will vary according to the surface properties of the NMPs and to their more or less great ability to undergo opsonisation phenomena, that vary significantly according to the animal species considered. Consequently, the choice of species potentially predictive of humans is particularly difficult: Dogs, for example, appear to be poorly relevant. The use of two animal species (rodent and non-rodent) may thus be inappropriate.

In the current state of knowledge, apart from NMPs used in medical imaging, little is known about their metabolism and excretion. In the case of polymers, it will be important to determine the nature of the degradation products, along with their elimination mechanism and kinetics. This determination is of great importance insofar

that these metabolites may possess certain toxic effects. It would also appear possible that the fate of certain nanoparticles in the body (quantum dots) may differ according to the dose administered: urinary elimination for low doses, transfer to liver, biliary excretion and elimination in stools for moderate doses (used in humans?) and finally liver storage in Kupfer cells (agglomerate and aggregate formation) for high doses.

The essential pharmacokinetic studies of NMPs must therefore be based on a case-by-case scientific approach, possibly by referring to the studies conducted on NMP already developed, by resolving any defects of these studies.

Observed effects should preferably be expressed per unit surface area to rather than unit of mass as is usually the case, as the smaller the particle, the greater the proportion of atoms exposed to the environment.

## **2. Safety pharmacology studies**

Studies adopting protocols adapted from pharmacological assessment (i.e. efficacy studies) for determining non-clinical safety have been extensively performed since the 1990's. These studies present the advantage of generally being conducted at a single dose, or at escalating doses administered over short periods, of being more flexible than toxicological studies and, most importantly, of applying to organs previously studied with insufficient precision for the functional aspects in toxicological studies. That is the case for the central nervous system, heart, kidneys, blood coagulation, etc.

There are no valid reasons to exempt NMPs from this approach, considering their impact (see below) on the cardiovascular system, lungs, kidneys and central nervous system. In the current state of knowledge, the use of the battery of tests recommended by the ICH S7A and S7B guidelines would appear to be acceptable. Assessment of the risk of QT interval prolongation appears necessary, though it remains to be determined whether the preliminary *in vitro* test on the effects on hERG current ( $I_{kr}$ ) expressed by HEK-293 human embryonic kidney cells is relevant and applicable to nanoparticle forms.

## **3. Toxicological studies**

### ***3.1 In vitro toxicity***

For the reasons indicated above (rapidity - ethics), and also in view of the absence of *in silico* data, it is highly recommended to develop and validate *in vitro* methods that are able to provide information on cytotoxicity, phagocytic capacity and macrophage activation, activation of complement pathways, biopersistence, generation of toxic free radicals, topical cutaneous, pulmonary and ocular tolerance (when these routes are used) etc. right from the prerequisite stage. Specific pharmacological tests, especially concerning the action on nerve cells and myocardial fibres, should be considered.

### ***3.2 Single dose toxicity***

Evaluation of single dose toxicity provides a wealth of information on the adverse effects of NMPs also administered as a single dose in man (imaging). These studies should be designed not as acute toxicity studies in which the endpoint is death, but as complete toxicity studies including evaluation of biochemical, haematological and histological parameters, as in repeated dose studies (extended single dose study). These studies could also be useful to rapidly compare active principles in nanoparticles and conventional forms, intended to be administered repeatedly in Man (anti-cancer drugs). The same will apply for local uses. When wishing to assess the acute toxicity of nanoparticle structures, unexpected events may occasionally be observed, for example: reduced toxicity

when the administered dose is increased, increased toxicity when particle size decreases. The role of agglomerate and aggregate formation and involvement of the reticuloendothelial system are probably related to these phenomena.

### **3.3 Repeated dose toxicity**

Repeated dose toxicity cannot be evaluated according to conventional study plans due to the structural and physicochemical differences of the particles, the animal species used, the indications and conditions of administration in therapeutics, etc. It will therefore be recommended to propose case-by-case protocols adapted to the above characteristics, reproducing human exposure conditions as closely as possible; standard protocols inducing massive exposure of the animals and consequently uninterpretable adverse effects should be excluded. Potential target organs or systems, due to the capacity of NMPs to cross physiological barriers should be investigated as a priority. In particular, potential targets include:

- Liver and organs of the reticulo-endothelial system (uptake),
- Kidney (e.g.: possibility of urolithiasis, tubular lesions),
- Central nervous system: various mechanisms have been proposed, especially passage across the blood-brain barrier, to evaluate the risk of neuronal degeneration. The hypothesis of brain exposure to certain toxic compounds, following a translocation phenomenon inducing the release of mediators causing inflammatory reactions, has been suggested in the development of neurodegenerative diseases.
- Reproductive organs (potential impairment of fertility),
- Cardiovascular system (e.g.: formation of aggregates),
- Development of inflammatory reactions, which appear to constitute a major risk for the respiratory tract, related to the formation of agglomerates and aggregates, due to its long-term consequences: cancer (DNA damage) and fibrosis (role of cytokines). Pulmonary inflammation also plays a major role in translocation phenomena, leading to exposure of other target organs, especially the brain. There is a high risk of induction of pulmonary intravascular macrophages, because of their phagocytic activity on NMPs or their microaggregates following intravenous administration. Major pulmonary haemodynamic disruptions are then possible. Scintigraphic and ultrasound imaging techniques are well-suited to for *in vivo* study of these phenomena, as is the use of conditional bioluminescent transgenic rodent models for mechanistic studies.

Evaluation of systemic exposure during animal studies in order to define safety margins for human exposures obviously remains to be investigated.

### **3.4 Particular toxicities**

Certain forms of toxicity may occur according to the characteristics of the NMP or according to the route of administration and it would be highly recommended to pay particular attention to these aspects.

#### **3.4.1 Immunotoxicity**

The immune response to a foreign substance introduced into the body can be globally divided into two compartments: the adaptive response specific

to the antigen introduced and the innate immune response not specific to the antigen. The structure and properties of NMPs suggest that these products are able to modify both of these types of immune response. The recognition of NMPs by "scavenger" receptors located on macrophages and polynuclear neutrophils can induce release of cytokines responsible for an inhalation pulmonary inflammatory response. NMPs have also been associated with the production of reactive oxygen species that may cause non-specific inflammatory responses. Moreover, matter in fine particle form is known to possess adjuvant properties that may exacerbate or modify the type of response to a given antigen (Th<sub>1</sub> response vs. Th<sub>2</sub>). In this case, this type of response may lead to hypersensitivity or allergic reactions. Finally, it is also possible that NMP scavenging or recognition by human dendritic cells could lead to immunosuppression, just as it is possible that NMPs are capable of modifying self antigens, thus inducing autoimmune responses.

In fact, in this sensitive area, the only clearly identified deleterious effect is the CARPA syndrome (C Activation Related Pseudo Allergy) observed in humans during colloid administration. It is characterised by fever, headaches, decreased arterial blood pressure, possibly leading to death and that cannot currently be predicted by conventional allergy testing. It would appear that all NMPs administered to date could potentially induce this syndrome in certain patients. The underlying mechanism of this effect is related to the production of complement fraction C3a and C5a, inducing massive cytokine release. Currently potential CARPA syndrome development is directly detected by complement activation tests conducted on patient serum or blood samples. Predictive tests are under development. It should be noted that the prevention of such a risk requires heavy and costly means (pre-treatment, hospital environment, etc.) that should be overcome by all possible means of prevention.

Consequently, assessing the immunotoxic potential of NMPs, in particular for drugs administered by inhalation is recommended. This assessment should involve adapted and validated methods. In the case of immunosuppressant potential detection, the methodology developed in the ICHS8 guidelines apply. This methodology is based on a "weight of evidence approach" taking into consideration the results obtained from repeat dose toxicology studies focusing on lymphoid organs and blood parameters, structure-activity relationship with immunotoxic effect, possible product accumulation in target immune system organs and the population to treat. Depending on the analysis of these results, a second assessment level may be implemented based on functional tests such as response to a T-dependent antigen. In the case of NMPs, the implementation of a functional test measuring the effects of these NMPs on specific response to an antigen is strongly recommended, due to their accumulation in macrophages or dendritic cells. NMP accumulation in lymphoid organs, that can be demonstrated by biodistribution studies, must also be considered as an alert signal.

Concerning hypersensitivity manifestations and in the current state, the most frequently used test for nanoparticle products (OECD guideline 429) is the determination of dermal sensitisation by the LLNA test (local lymph node assay) in mice, for example for the various forms of titanium oxide used in cosmetology. Nevertheless, results must be critically interpreted, particularly in the case of NMPs intended for pulmonary use. In the case of protein- or DNA-bound NMPs, particular attention must be paid to modifications of the immunogenicity of the products associated with the NMPs. The development of cellular models using human cell lines, under defined conditions and after product physicochemical characterisation, with particular focus on effects on macrophages, granulocytes and dendritic

cells, should be strongly encouraged, in particular to study the effects of NMPs on cytokine and immune system mediator production. The development of recent PET imaging methods using <sup>18</sup>F-labelled 2'-deoxycytidine derivatives to assess the immune system and lymphoid organs should also be encouraged.

#### *3.4.2 Risks related to the formation of agglomerates*

This is a classical risk, identified right from the use of the first NMPs (liposomes). The formation of agglomerates can affect various territories in the body, especially in smaller vessels (peripheral microcirculation, cerebral vessels, etc.) by inducing embolic phenomena.

This potential should be evaluated by appropriate animal studies, focused on histological examinations.

#### *3.4.3 Local effects*

Nanoparticle systems can induce severe irritation and inflammation phenomena via direct mechanisms or mechanisms mediated by signalling pathways. This should be investigated for all routes of administration, particularly topical routes: skin, eye, and especially lung, as indicated above. It is reasonable to propose that OECD guidelines 404 and 405 can be applied to evaluation of the irritating potential on the skin and the eye after single administration in rabbits. The preferred technique to assess local effects on the lung consists of intratracheal administration of the test substance in rats followed by bronchoalveolar lavage (evaluation of biological markers of inflammation in the lavage fluid), evaluation of cellular proliferation and histological examination. This type of study could be associated with evaluation of reversibility of the observed phenomena (after one to four weeks for example).

The haemolytic potential by IV administration of NMPs should be evaluated, if this route of administration is used in therapeutics.

### **3.5 Reproduction toxicity**

No published data are currently available concerning the potential effects of nanoparticles on reproduction, fertility and their teratogenicity, and these aspects need to be evaluated. For example, passage of NMPs across the foeto-placental barrier makes evaluation of embryofetal toxicity and the teratogenic potential essential. The protocols described in current guidelines should therefore be used, but may need to be adapted to NMPs. Maternotoxic and teratogenic effects have been described for medical imaging products (probably due to iron overload).

### **3.6 Genotoxicity**

#### *3.6.1 Potential mechanism of action*

Although the ability of nanoparticles to cross cell membranes has been established, much less is known about their ability to reach the cell nucleus at the appropriate time of the cell cycle to interact directly on DNA, especially during cell division when the nuclear envelope is lost. Thus, direct and indirect primary genotoxic effects, but also secondary genotoxic effects, could occur:

a) Direct primary effects: it is conceivable that:

- Nanoparticle penetrate the nucleus and interact directly with DNA

- Nanoparticles produce free radicals that induce DNA lesions
- Nanoparticles disrupt chromosome segregation during mitosis (aneugenic potential). Indeed, due to their nanometric size, it is commonly accepted that nanoparticles are able to directly interact and possibly interfere with cell components of comparable size, such as nucleosomes, microtubules, actin filaments and centrosomes. Thus, interference with these structures could lead to dysfunction of cell division and disruption of cell traffic.

b) Indirect primary effects: nanoparticles could cause:

- Antioxidant depletion, either directly increasing the number of endogenous DNA oxidative lesions, or that of DNA oxidative lesions related to mitochondrial respiratory chain disruptions, leading to the production of reactive oxygen species (ROS) and to an interruption in ATP synthesis
- Inhibition of DNA repair

c) Secondary effects

These effects would appear to be mainly due to inflammation, via oxidative compounds produced in particular by endocytosis and/or phagocytosis of nanoparticles. Indeed, nanoparticle can generate oxidative stress and inflammatory responses, which may also potentially induce DNA lesions. The radical species formed (in particular the °OH ion) may also interact with polyunsaturated fatty acids, thus initiating a lipid peroxidation process. This results in the formation of aldehydes capable of producing DNA adducts.

### 3.6.2 Assessment methods

#### 3.6.2.1 General Remarks:

Concerning the genotoxicity assessment, it will be emphasized once more on the need to gain a robust knowledge on the structure and properties of the nanoparticle form in excipients and culture media used in the tests because its behaviour will be modified. These informations can be also necessary for a better correlation of the results of *in vitro* tests and for a better extrapolation of *in vitro* to *in vivo* results. If a nanoparticle is intended to carry a given substance (medicinal product, gene...), and if the vector and assembled form possess different physicochemical properties, the both forms must be assessed for their genotoxic potential, unless if it is possible to demonstrate that during exposure both forms will be assessed.

There are currently no reasons to formally exclude, for NMPs, a basic battery of tests as recommended for conventional drugs. Considering the knowledge regarding the main mechanisms involved in the genotoxic effects of some nanoparticles, usual models in this battery, such as bacterial tests, appear to be of lower relevance than other tests.

In the case of *in vitro* tests, the cell type used should, whenever possible, be representative of the *in vivo* target organ in terms of toxicity and/or most exposed organ (primary exposed organs and/or organs exposed after translocation), that will be defined according to current knowledge of the route of exposure, absorption levels and degree of translocation (pharmacotoxicokinetic studies).

The use of specific models, such as reconstituted human skin, intestinal cell, pulmonary cell or co-culture models (e.g.: pulmonary cells + neutrophils, etc.)

may, in certain cases, be considered in order to use models more closely mimicking human exposure conditions.

If a primary cell line or continuous mammal cell line is used, certain phenotypic expression data for this cell line must be documented, in particular, its p53 status and ability to deal with reactive oxygen species (SOD, GSH/GSR, GST, GPX, etc.). Moreover, as the intra- and extracellular flow of nanoparticle structures are determined by the cell's endocytosis and exocytosis abilities, these must be documented for all cell lines used.

#### 3.6.2.2. *In vitro* tests

As for a conventional medicine, a battery of tests is also necessary for NMPs, leading to an as large as possible evaluation of the genotoxic spectrum. Currently, there is no consensus on the best way to carry out this evaluation. The purpose of this recommendation is to provide a synthetic overview and to propose a reviewable approach.

##### 3.6.2.2.1. Gene mutation tests

Concerning the gene mutation tests, bacterial tests are insufficient to ensure that bacterial DNA is exposed, due to the lack of demonstration to date of the ability of nanoparticles to penetrate bacterial cell walls and membranes. Furthermore, certain mechanisms of genotoxic action of nanoparticles are caused by interaction with mitochondria, while this mechanism cannot be demonstrated on bacteria. These models may, however, be useful to demonstrate mutagenic effects from impurities, salting out products and/or degradation products. For gene mutation tests conducted in mammalian cells, the L5178Y, CHO and V79 cell lines routinely used for studying this type of effect are considered as deficient in phenotypic expression of protein p53 and some are also deficient in the expression of certain free radical detoxification enzymes. These deficiencies could impede the interpretation of results obtained with these cell models. The non-human origin of these cell lines could also further non specific and/or non valid results for human cells. These *in vitro* models are currently questioned because of the high percentage of positive results, when compared with carcinogenicity studies in rodents, and their relevance is still a question under discussion, as well for small molecules. So, no validated models are currently regarded as acceptable for the assessment of gene mutation on mammal cells for NMPs.

##### 3.6.2.2.2. Chromosomal mutation tests

Concerning chromosomal mutation tests, it has been shown that certain nanoparticles are capable of demonstrating both clastogenic and aneugenic effects. The *in vitro* micro-nucleus test appears to be well-suited for the demonstration of these two types of effect. For the above-mentioned reasons, this test should be conducted using primarily human cell lines, such as human lymphocytes. Certain protocols use cytochalasin B to identify actively dividing cells. Cytochalasin B acts by inhibiting actin polymerisation, which is involved in certain endocytosis steps. However, cytochalasin B should be used under conditions that do not disrupt nanoparticle endocytosis and/or exocytosis (e.g. some hours after the treatment).

##### 3.6.2.2.3. Primary DNA damage tests

Insofar that the use of gene mutation test is a question under discussion, an *in vitro* primary DNA damage test could provide useful information for a decision to assess the genotoxic risk, in particular concerning the decision to perform *in vivo* tests. For the *in vitro* demonstration of primary DNA lesions, the comet

test appears to be well-suited, on condition that a relevant evaluation of cytotoxicity is made (in particular by overcoming any apoptotic or necrotic effects). For example, it can be useful to assess cytotoxicity after a period corresponding to at least one cycle of cell division, to avoid that genotoxic effects be taken into account at irrelevant lethal concentrations. Moreover, as a significant proportion of nanoparticle-induced genotoxic effects is likely to be related to the production of activated oxygen forms, it seems necessary to supplement the conventional protocol by using enzymes for demonstrating oxidative DNA lesions, such as by treating the cells with fpg or hOGG1 proteins.

In all cases, when selecting the doses to use, care must be taken to avoid inducing effects non-representative of realistic human exposure. As oxidative stress, which plays a key role in nanoparticle-induced genotoxic effects, is in part related to surface properties, it appears preferable, particularly for monolayer cell cultures, to express the dose level per unit area (e.g.:  $\mu\text{g}/\text{cm}^2$ ) rather than as a mass concentration.

Information concerning an inflammatory response ( $\text{TNF}\alpha$ , IL6, etc.) induced by NMPs, with particular reference to the choice of dose and for the interpretation of results, may be useful to extrapolate the studied dose level to human exposure levels.

#### 3.6.2.3. *In vivo* tests

When *in vivo* tests must be conducted, routes and conditions of exposure should be selected to reflect clinical conditions in terms of exposure levels, administration frequency and cytotoxicity, but also in terms of the induction of inflammatory phenomena. In particular, the recruitment of cells involved in inflammation, such as macrophages and neutrophils that may have their own specific effects through the production of free radical forms, or by interfering with repair mechanisms, must be rigorously taken into consideration.

Depending on the type of therapeutic use (route of administration in particular), of pharmaco/toxicokinetic data and of the results of *in vitro* tests, the *in vivo* micronucleus test (e.g.: on bone marrow, colon, circulating lymphocytes, etc.) and/or the *in vivo* comet test are recommended and should be conducted on rodents.

It is recommended, when it is feasible and scientifically relevant, to conduct these tests on animals comparable to animals used in short-term repeated dose toxicity studies or in the course of these studies.

#### 3.6.3. Strategy of the genotoxic potential evaluation

The basic battery of tests for the determination of an NMP's genotoxic potential must include at least an *in vitro* micronucleus test, an *in vitro* comet test on mammalian cell cultures and an *in vivo* test.

a) If the *in vitro* micronucleus and comet tests are negative, an *in vivo* micronucleus test should be performed on the most highly exposed organ(s) to investigate if secondary effects on DNA can occur *in vivo*.

b) If the *in vitro* comet test is positive, an *in vivo* comet test should be performed on the target organ and/or the most highly exposed organ(s), unless if it can be demonstrated that the indirect primary effects on DNA do not occur *in vivo* under therapeutic conditions.

c) If the *in vitro* micronucleus test is positive or if both *in vitro* tests are positive, an *in vivo* micronucleus test and an *in vivo* comet test should be

performed on the target organ and/or on the most highly exposed organ(s). It is recommended to conduct both tests on the same animals.

When making the final decision concerning the genotoxic potential of an NMP, the weight of evidence shall be determined based on the mode of action at those doses having demonstrated a genotoxic effect. In this case, all additional and relevant data to clarify this mode of action should be taken into account.

### **3.7 Carcinogenic potential**

Evaluation of the experimental carcinogenic potential of NMPs is currently a controversial issue:

- On the one hand, it is clear that, in view of their structure, their potential to induce DNA damage and inflammatory reactions and their bioaccumulation, NMPs could induce tumours, especially lung tumours.
- On the other hand, the protocols recommended by guidelines are complex, time-consuming, and poorly adapted to exposure to nanoparticles (metrology, control of exposure, etc.). Furthermore, carcinogenicity studies do not appear to be necessary in view of the current applications of NMPs (single dose in medical imaging, vectorization of anti-cancer drugs).

Consequently, the assessment of an NMP's carcinogenic potential is only justified after careful consideration of the potential hazard and risk assessment, in order to avoid drawing hasty and inappropriate conclusions. In the current state of knowledge, and despite the difficulties that will inevitably be encountered, it would appear that conventional two-year study protocols on rodents are acceptable for the Agencies in charge of drug assessment. Demonstration of the consistency of administered product characteristics shall most certainly be demanded, in particular for inhalation studies. Assessment of exposure appears to concern only the vectorised active principle, possibly compared with that obtained for the non-vectorised active principle, when achievable. An adaptation of current protocols is highly recommended: for example shorter studies, studies with a limited number of administrations, use of transgenic mice, etc. Particularly relevant evaluation of the genotoxic potential of NMPs would obviously be very useful as part of risk assessment.

## **D. CONCLUSION**

The recommendations formulated in this document are based on the following concept: evaluation of the safety of NMPs, taking into account scientific and practical considerations such as the need to be immediately operational, must not fundamentally differ from the conventional strategy of safety evaluation of medicinal products. However, the methods of this evaluation must be adapted when necessary and the results must be expressed in relation with the particular characteristics of the nanoparticle structure.

Nevertheless, a much longer term view cannot be excluded and it will also be recommended to apply the conclusions of the document "Nanotechnology: A report of the US FDA Nanotechnology Task Force" published on 25 July 2007, which proposed the following longterm objectives to the FDA:

- Evaluate the adequacy of current testing approaches to assess safety, effectiveness, and quality of products that use nanoscale materials;
- Promote and participate in the development of characterization methods and standards for nanoscale materials;

- Promote and participate in the development of models for the behaviour of nanoscale particles *in vitro* and *in vivo*.

This ambitious objective could be confided by Afssaps to a Task Force composed of academic scientists, members of regulatory authorities and obviously manufacturers specialized in this field.

**N.B.** : This document is not designed to formulate recommendations for the evaluation of environmental toxicity.