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ANIMAL TEST ALTERNATIVES IN METAL TOXICOLOGY RESEARCH

A study by “in vitro” cellular systems

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INTRODUCTION

1.1. Alternative Testing Methods

Regulatory toxicity testing in animals raised a number of scientific, humanitarian, legislative, practical and economic questions and conflicts. For example, while the use of animal procedures in identifying the potential hazards represented by chemicals and products of many kinds is required by various laws and regulations, there are other laws, of no less importance, which are intended to protect laboratory animals from unnecessary pain, suffering, distress or lasting harm (Balls, 1995a). They stipulate that the use of laboratory animals should only be permitted when it can be justified as necessary on strong scientific grounds. However, the scientific basis of many animal tests themselves, and, in particular, of the ways whereby the data they provide are currently applied in human risk assessment, is weak. In addition, the present dependence of hazard prediction on animal tests requires considerable human and economic resources, as a result of which only a very small proportion of the chemicals which might threaten the well- being of humans, and of the environment in general, have been subjected to anything approaching a scientifically- satisfactory evaluation (Balls, 1995b).

Non- animal tests and testing strategies can offer solutions to many of the problems caused by the current over- reliance on the routine, check- list application of animal tests. They can offer a more- mechanistic basis for understanding toxic effects at the systemic, organ, cellular and molecular levels (Sabbioni and Balls, 1995). When human cells and tissues are used, or modelling is based on human experience and responses, the problem of species differences, which severely limits the relevance of animal tests, can be avoided. The change of emphasis from “in vivo” to “in vitro”

approaches must also embrace the increasing use of properly- obtained and safely-maintained “human” rather than “animal”, cells and tissues. Well- designed “in vitro” studies can also assist with the selection of the most- appropriate laboratory animal species for any subsequent, necessary use as a model for man (Fentem and Balls, 1994).

Non- animal methods offer an escape from many of the ethical and legal conflicts inherent in current practices. Often, they also offer the possibility of more-manageable, less time- consuming and less expensive testing strategies, so that the practical and economic limitations which currently prevent the adequate testing of many chemicals and products can be overcome (Balls, 1995a).

1.2 The present research in the context of ECVAM activities: the IMETOX project.

Cell culture technology and accompanying “in vitro” cytotoxicology, is an important newly developing discipline of modern toxicology and is gaining increasing acceptance in the field. In this context, it is useful to separate cell culture methods in toxicology into two different areas of applications which complement each other:

a) study of mechanisms of toxic effects, including effects on cell membranes, DNA, protein and lipid production as well as biotransformation of chemicals

b) testing, where the qualitative and quantitative toxicity of previous unclassified chemicals is estimated (Sabbioni and Balls, 1995).

These aspects are also key points of the research activities carried out in the context of IMETOX project (“In vitro” Metal TOXicology) (Sabbioni, 1998) at ECVAM (European Centre for the Validation of Alternative Methods) which foresees the use of “in vitro” toxicity testing of trace metals in different toxicological areas such as

embryotoxicity, haematotoxicity, nephrotoxicity, neurotoxicity, metabolism-mediated toxicity (Balls and Fentem, 1997). Since this work is a part of the IMETOX herein few essentials on the aims and activities concerning ECVAM and IMETOX projects as reported below.

ECVAM is a Unit of the JRC at Ispra created by the European Commission in October 1991 to implement the Directive 86/ 609/ EEC regarding the Protection of Animals Used for Experimental and Other Scientific Purposes (O. J. EEC, 1986) which is directed to reduce, refine and replace the use of laboratory animals in accordance with the Three Rs concept of Russel and Burch (Russel and Burch, 1959). The main task of ECVAM is the co-ordination of the validation of alternative testing methods at the EU level; and the promotion of dialogue among legislators, industries, biomedical scientists, consumer organisations and animal welfare groups with a view to the regulatory acceptance of non- animal, regulatory tests that is based on the formal validation which includes three phases (Figure 1.1):

The IMETOX project aims at integrating the aspect of toxicity of metal compounds with current ECVAM activities related to the in- house development of “in vitro” tests and prevalidation/ validation studies. Key aspect of the project is the study of the response to metal exposure on cellular level (uptake, intracellular distribution, biotransformation and cytotoxicity of metals) in relation to endpoints at molecular level. Exposure of cells to low doses of specific or combined mixtures of different metal compounds and long term cell culture testing are other main aspects considered (Sabbioni and Balls, 1995).

Selection of metal compounds to be tested takes into account the existing “in vivo” knowledge on their metabolism, mechanisms of toxicity, induced toxicological effects

which are relevant to environmental and occupational exposure, drug therapy, use of food and cosmetics, implantation of biomedical devices. The choice of cell lines for toxicity testing and the selection of metal compounds investigated in this work are placed in this context.

Figure 1.1 STAGES IN THE EVOLUTION OF REGULATORY TESTS

1. TEST DEVELOPMENT (Laboratory of Origin)
· Purpose and need for test
· Derivation of method
· Application to appropriate chemicals
· Prediction model for application of results

2. PREVALIDATION (Informal Interlaboratory Study)
· Confirmation of interlaboratory transferability
· Optimisation of prediction model

3. VALIDATION (Formal, Interlaboratory Study, including a Blind Trial)
· Study design
· Selection of tests and laboratories
· Selection and distribution of chemicals
· Data collection and analysis
· Assessment of outcome according to prediction model
· Evaluation and proposal of next steps

4. INDEPENDENT ASSESSMENT OF STUDY AND PROPOSALS

5. PROGRESSION TOWARD REGULATORY ACCEPTANCE

a: from Balls and Fentem, 1997

1.3. Metal toxicity and human health

There are several reasons for the great interest in the biological effects of trace metals from the aspect of their toxicity. The concept of the ”health impact” of trace metals can be discussed characterising the optimal doses of metals responsible for the essential actions in the organism, and on the other side, the doses able to induce toxic effects in the organism as a consequence of excessive expositions to trace metals. Bertrand (Bertrand, 1912) studied how high concentrations of trace metals present in the environment represent a potential health risk for the human body. In particular, the concentrations of certain trace metals or their compounds in the environment and their uptake by the organism may, under certain conditions, reach levels incompatible with human health. For some time, these adverse conditions were confined to some pre- determined areas of the geosphere, where they have been existing without any significant interference through human activity. It has been found important to consider the relation between the exposure to single chemical agents and the relative human risk. Some trace metals are essential elements for reproduction, development and growth of the organism (Mertz, 1981). Intake of low doses of these metals can alter the optimal biologic function of the body. The biological function can be restored when normal doses of these elements in the body occur (Sabbioni, 1984). On the other hand high exposure to these essential elements can produce harmful effects to human health. This concept has been expressed by the dose- relationship curve (Bertrand, 1912) and represents still the basis of the modern trace metal toxicology.

For some time the hazards connected with man- made changes in the environment were limited to selected groups of individuals. Toxic effects were confined to relatively narrow groups of individuals near the source of the toxic metals, particularly at the workplace (Browning, 1969). During the past decades the problem of trace metal toxicity has exceed

the limits of geochemically or professionally exposed subjects. The broad scope of environmental changes, mineral pollution of air, water and soil, connected with the growth of industry, urbanisation, development of transport and the use of agrochemicals and other changes affecting human and animal nutrition, have caused far- reaching changes in the exposure of large population groups to certain trace metals (Sabbioni et al., 1983; Sabbioni et al, 1985 and Sabbioni, 1985a). The situation can be serious not only because toxic metals can remotely act from their original source and affect large population groups, but also because the changed uptake is not necessarily confined to one metal only but could involve a simultaneous uptake of other trace metals (interrelation among elements). Change of the exposure of living organisms to mercury and selenium at the same time by human activities can lead to unexpected toxic results (Moffitt and Clary 1974). Taking into account the above mentioned considerations the setting of standard health criteria to regulate the emission of trace metals in the environment and to establish maximum levels of exposure compatible with human health is necessary. Although this action involves political decisions, the determination of the criteria on which these decisions are based is a scientific problem that requires a multidisciplinary approach (Sabbioni et al., 1990).

The risk that large population groups could be exposed to trace metals in a form and dose incompatible with good health and that this could arise from human activity seems to reveal completely new problems connected with trace metal toxicology. First of all, in addition to clinical manifestations of acute illness, much more should be known about sub-clinical adverse effects of the inadequate exposure of trace metals. For example, it is growing evident that in highly developed countries occupational exposure to toxic metals is decreasing, in some cases approaching the levels of environmental exposure. Thus, certain trace metals and their compounds can be involved in some chronic human diseases, affecting systems which are sensitive to low dose exposure over long periods such as

nervous-, reproductive-, immune- and cardiovascular systems (Alessio, 1992). In this contest The European Commission promoted an action programme concerning the environmental protection (O. J. EEC ,1977; O. J. EEC, 1980; Vagn H. 1993). The importance of the exposure of trace metals is demonstrated by cases of intoxication of general population by cadmium and mercury (Kitamura, 1978; Kiragishi, 1981). These events were caused by scientific ignorance related to toxicological effects induced by long-term low doses exposure of species of both metals.

The health impact of trace metals is complicated by the fact that people can be exposed to different chemical forms of the same metal like simple inorganic forms, complex inorganic forms and metallorganic forms (Sabbioni et al., 1987). The different forms of the same compound interact differently with cell components inducing different toxicological effects (speciation) (Sabbioni et al. 1990). Metabolic- speciation studies on inorganic arsenic have proved clear evidence that no animal species, among those so far investigated, is an appropriate model for arsenic metabolism in humans (Sabbioni et al., 1985b). The different metabolic patterns and toxicological effects of arsenic “in vivo” and “in vitro” as inorganic forms (trivalent and pentavalent ions) compared to organo- arsenic species (e. g. arsenobetaine in seafood) are examples of how different metal species of a same metal affect its toxicity (Bertolero, 1987 and Sabbioni, 1991). Inorganic arsenic (As(III) and As(V)) were found to be more toxic than organo arsenic species such as (monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and trimethylarsenoxide (TMAO), arsenobetaine and arsenocholine) (Sabbioni and Balls, 1995; Cocco, 1999). Chromium is another example is essential when its oxidation state is (III) but is toxic when is present as chromium (VI) (Mertz, 1972). Moreover, it has become clear that several metals undergo biotransformations in mammalian tissues and that the metabolism has important implications in trace metal human toxicology, pharmacology and occupational

health (Manzo et al., 1992). The knowledge of the metabolism of the different chemical species of the trace metals is essential to describe the detoxification process of trace metals. In this process the initial toxicity of a metallic compound is reduced before its eventual elimination. Two examples are methyl mercury and arsenic. Mammals are able to de- methylate Hg and methylate As generating low toxic metabolites (Sabbioni et al., 1990; Marafante et al., 1982). These results are important for the researches aimed at developing metabolic models that describes quantitatively the toxicokinetics of absorption, of distribution, the transformation and excretion of the different chemical forms of trace metals in the organism. These models (Lutz et al., 1987) are an important aspect to understand the link between exposition models and models for risk analysis in order to predict the accumulation of metals in target organs.

Therefore, it can be concluded that some aspects of trace metal toxicology should have priority and that certain research needs can be formulated concerning the present status of the problem:

a) there is a need to provide more systematic information on the toxicological effects of trace metals following chronic exposure to levels which are normally present in polluted environments (long term- low level exposure)(Sabbioni, 1981).

b) there is a need for metabolic studies as a basis for interpretation of metal toxicity. The potential of metals to cause toxicity has traditionally be regarded as a function of dose and potency of the metal itself. Since the effects of metals are related to their concentration at specific target sites, metabolic data are also important in interpreting toxicological findings. This includes also metabolic- speciation studiesat intracellular and molecular level.

c) the assessment of risk to human health associated with exposure to trace metals involves an integrated approach which is based on data from “in vivo” studies on animals, clinical

studies on humans and "in vitro" toxicity testing methods. These latter can contribute to give fundamental information on the ranking of metals for their potency, the identification of both mechanisms of toxic action of metals and of potential target organ toxicity (Frazier, 1992).

1.4. Objectives of the present research

The purposes of this work are connected with the aim of the IMETOX project ("In vitro" Metal TOXicology). In particular, the research of this thesis has been carried out in the context of four different toxicological areas (Table 1.1) such as:

a) Dermatotoxicity. Investigations carried out concern a screening of the cytotoxic effect of 51 metal compounds in human keratinocytes (HaCaT immortalised cell line) exposed to a fixed dose of 100μM of individual metal compound and the setting of dose- effect relationships for selected metal compounds including "newer" potential environmental pollutants such as different chemical forms of Pt.
b) Neurotoxicity. A systematic study on the cytotoxic response of 14 metal compounds was performed on engineered modified rat pheochromocytoma PC12 cell line by exposure to a fixed dose (100μM) of cells to individual metal compounds.
c) Embryotoxicity (cardiotoxicity). A preliminary study was focused on the effect of methyl mercury at different stage of differentiation of embryonic stem cells D3 into contracting myocardiac cells.
d) Carcinogenic potential. Uptake, intracellular repartition, cytotoxic effects and morphological transformation of inorganic species (As (III) and As (V)) as well as organo- arsenic compounds (arsenobetaine (Asβ), arsenocholine (AsCh)) in BALB/3T3 cell lines.

Table 1.1- Objectives and type of studies of the present research.

Cell line	Type of end- point	Expected contribution as "in vitro" model	Type of study carried out
HaCaT	CFE Test	Development of a cellular model for metal dermatotoxicity	Screening toxicity testing at a fixed dose. Determination of the IC_{50} for selected metals
PC12	MTT Test	Development of innovative and sensitive cellular model for metal neurotoxicity	Screening toxicity testing at fixed doses
ES- D3	Beating measurements	Development of innovative and sensitive cellular model for embryonic cardiotoxicity	Effect of MeHg on differentiation in contractile cells
BALB/3T3	CFE- Morphological Transformation (Foci formation)	Development of "in vitro" model for the potential carcinogenicity of metals	Effect of different metal compounds on the formation of type III foci

LITERATURE SURVEY

2.1. “In vitro” tests and their growing importance in metal toxicology

There is growing and increasing interest in the use of cell cultures in metal toxicology research as simple and rapid “in vitro” assays that can act as preliminary screens, possibly reduce and replace the need for animal tests, and as a tool in understanding the mechanisms of metal- induced cytotoxicity at subcellular and molecular levels. Figure 2.1 summarises advantages and disadvantages of the use of cell cultures in toxicological studies connected with the exposure to xenobiotics, including trace metals.

Figure 2.1- Advantages and disadvantages in the use of “in vitro” toxicity testing.

ADVANTAGES	**DISADVANTAGES**
- The system is highly reproducible. - Good control of the chemical-physical conditions. - Quick response statistically valuable. - The reversibility of the effects can be easily checked out. - Characterisation and homogeneity of the sample. - The cellular and molecular mechanisms can be easily explored. - The damage can be easily identified. - Human cells can be used. - “In vitro” metabolism is limited. But in its limitation is more constant than the “in vivo” one. - The speed of the “in vitro” cellular growth is higher than the “in vivo” one, with high sensibility of response. - The work can be done with little quantities of sample. - Technically, the cell cultures are easy to work with. - The cell cultures are less expensive and quicker than the experimentation with animals. - The cell cultures can reduce the use of the animals in the experiments.	- The system is too easy in comparison with the “in vivo” one. - The toxic effects produced by hormones or by the central nervous system can’t be studied. - The toxicity mechanism can be studied only using specific cell lines. - Instability: some cell lines are aneuploids. - The different concentrations of the substances involved in the experiments are difficult to be chosen for the “in vitro” experiments in comparison to the “in vivo” one. - The exposure conditions of the “in vitro” cultures to different compounds, can create problems that derive from the chemical-physical properties of the compounds. Compounds that can interact with the medium components. - The tissue-specific interaction between the cells disappear. - The toxicity can be the result of the damage of communications between cells and not the result of a cell damage.

The knowledge of the trace metal metabolism is not sufficient per se to establish dose-effect relationships as basis to estimate the toxicological risk connected to trace metal exposure. There is also the need of correlating pathological processes, deriving from metal exposures, to specific characteristics of tissues and cells from where these processes take place. In particular, the use of cellular systems of mammalian origin in combination with nuclear and radioanalytical techniques such as neutron activation analysis and the use of radiotracers of high specific radioactivity can be considered of particularly utility for investigating the correlations between the metabolic pathways of the metals (uptake, intracellular distribution, binding with molecular components) and the toxicological effects induced by trace metals “in vitro” (Sabbioni and Balls, 1995).

The use of radiotracers with high specific radioactivity is a potent tool in analytical toxicology related to research on trace metals carried out by cell cultures. Main advantages are:

a) possibility to label “low doses” of trace metals under conditions adapted for biochemical purposes, e. g. to follow the metabolic patterns of low levels of metals in different cell fractions and molecular components.

b) their addition to cell culture medium that does not induce significant alteration of the concentration of the stable metal already present in the system, and permits to delineate the “normal physiological metabolism” in the cell, e. g. to establish the distribution among cellular components and the binding with biomolecules carried out at concentrations far from saturation phenomena at binding sites.

c) the simultaneous administration, detection and measurement in cellular systems allow the study of combined mixtures of trace metals, gaining information on synergistic and antagonistic effects between essential and toxic elements.

2.1.1. Screening studies

"In vitro" screening investigations on the cytotoxicity of metals are important not only to establish the ranking of toxicity of metal species present in the environment in wide sense (general environment, workplace, industrial processes and use of metals in biomedical field as drugs). Such kind of studies are of great scientific value because of the possibility of performing experiments exposing cells to a mixture of metal compounds (aspect of exposure to combined exposure). For example, biomaterials implanted in the organism (e.g. dental implants) can release metals when such devices come into contact with biological fluids, generating mixtures of metals. A second example of combined exposure concerns hard metal exposure. Its cytotoxicity mechanisms can be studied with alternative methods (cell cultures). Hard metal is a metal alloy made of tungsten carbide (W, 70%-95%) with different percentages of other metals like titanium, tantalum and vanadium as carbides with cobalt as binder (5%- 25%). The composition of these alloys can change according to their programmed use, in particular in the metal mechanic industry, in automobile and in military field. Professionally exposed workers to those powders react with asthma and pulmonary fibrosis (or "hard metal disease") (Sabbioni et al., 1994). This latter disease was found not to be correlated to the anagraphic age but correlated to the time spent working and the exposure dose. The origin of this disease is immunotoxic: the immune sequence is primed by cobalt that acts as hapten, although synergistic/ antagonistic effects are also possible due to the simultaneous exposure to other metals (Sabbioni et al., 1994).

Thus, since the extrapolation of animal data to humans is problematic (e.g. due to qualitative and quantitative interspecies differences in the metabolism and toxicity of a metal compound (Blaauboer, 1995).

Screening studies by cellular models seem suitable as complements to risk assessment.

2.1.2. Mechanistic studies

The mechanisms of action of metals are related to their biokinetics inside the cells. Many of the characteristics of metals that determine their kinetics and dispositions are quite different form those that control the kinetics and disposition of volatile organic chemicals. Key aspects include binding to macromolecules with respect to distribution, sequestration and mechanisms of action of a metal; mechanisms of metal uptake by cells; the possibility of metal- metal interactions; and, for some metals, the long biological half life in the body. Since this matter is too wide and a deep review of the aspects related to the mechanisms of action of metals is not the scope of this work we limit our considerations to some bioinorganic aspects and to basic processes involved in the detoxication mechanisms involving some metals.

Bioinorganic aspects. Some metals form oxyanions in biological solution. Those that resemble phosphate structurally (for example vanadate and arsenate), use the phosphate transport system to penetrate cells (Huang and Lee, 1996), while those that resemble sulfate (for example chromate, molybdate and selenate), use the sulfate transport system (Cardin and Mason, 1975; Wetterhahn- Jennette, 1981). Certain cations can penetrate into cells through voltage- sensitive calcium channels. Also neutral, lipid- soluble complexes diffuse into cells. Diffusion does not preclude the parallel operation of a carrier uptake mechanism. Certain other metals may enter cells by facilitated transport in the form of organic complexes (Clarkson, 1993).

In this context, toxic and essential metals may compete with each other for binding sites on proteins, including membrane transport proteins and enzymes. Interactions of metals at enzyme active and structural sites, and at metabolic control points, are varied, complex,

and often difficult to predict “a priori”. Nevertheless, such interactions may be integral to the mechanism(s) of action of a metal (Fridovich, 1975).

Since the binding of a metal to a protein involves specific binding sites, it is always potentially capacity- limited (O’ Flaherty et al., 1996). The simplest way to characterise capacity- limited binding is by means of a Michaelis- Menten type of expression, with binding determined by the maximum binding capacity and the half- saturation constant. Inducibility of binding proteins may be age dependent. A study on high- affinity metal-binding proteins has shown that they are important not only in control of tissue distribution but also of target organ toxicity (Squibb, 1996).

Detoxication mechanisms involving trace metals. The cell protects itself against metal toxicity by different mechanisms.

Binding of metals to proteins may represent a detoxication mechanism. The protein metallothionein is one of the most intensively studied metal- binding protein, particularly because it is able to bind essential elements such as zinc and toxic elements such as cadmium and mercury, in liver and kidney (Palmiter, 1995). Binding to metallothionein is at least partly responsible for the extended residence time and accumulation of cadmium in the kidney (Squibb, 1996).

The binding to proteins includes also low- molecular- weight ligands including carboxyl-, amino-, and sulphydryl- containing organic compounds and chloride, water, hydroxyl, and bicarbonate groups. Many of these complexes are quite labile. The pH, ionic strength, and composition of body fluids determine the range of metal species present. The structure of and net charge on the metal complex will, in turn, determine the ease of its transfer into cells as well as the route(s) and mechanisms of its excretion (Palmiter, 1995).

In addition to the introduction of specific sequestring proteins (e.g. metallothionein) main processes involved in detoxication processes include oxidation/ reduction or alkylation/

dealkylation of toxic metal compounds. For example, chromium (Cr^{6+}) is reduced to Cr^{3+} intracellularly (De Flora and Wetterhahn, 1989), while inorganic arsenic, As^{5+}, is reduced to As^{3+}, which in turn is methylated to monomethyl, dimethyl, and trimethyl derivatives. Not all species are able to methylate arsenc at the same extent (Vahter, 1994). In addition, most experimental animal species excrete arsenic as dimethylarsinic acid (DMA), while humans excrete also monomehtylarsonic acid (MMA).

2.1.3. Contribution to risk assessment

One major goal of "in vitro" metal toxicological research addressed to regulatory work is to provide scientific information on exposure and health risk assessment (Salama et al., 1999). Risk assessment can be defined as a process used to determine the probability that adverse or abnormal effects are associated with exposure to a chemical (e.g. heavy metals), physical or biological agent under particular conditions of exposure. The risk assessment process usually involves hazard identification, dose- response assessment, exposure assessment, and risk characterisation. Risk management, on the other hand, is the process that applies information obtained through the risk assessment process to determine whether the assessed risk could be reduced and, if so, to what extent (Stingele, 2000). In this context, the "in vitro" metal toxicology by cell cultures represents an important contribution to the risk assessment process. For example, many approaches and techniques have been developed for monitoring human populations that have been exposed to environmental mutagens (Hulka et al., 1990). The traditional approach has been to use readily available cells to document biomarker of effects (Salama et al., 1999). However, biomarkers studies are still not generating the type of reliable information needed for precise risk assessment. Some of the problems are due to inconsistent observation of biological effects from similarly exposed populations, lack of predictable dose- response

relationship and existence of interindividual variations in response to exposure (Au et al., 1998). “In vitro” studies by c<ell cultures can contribute to elucidate whether the dose-response to specific exposure conditions are reproducible, to identify the more sensitive and specific tissue target, and to gain more precise prediction of health effects (Salama et al., 1999).

The tests done in our study were not only developed as “toxicological tests” for specific toxicological area (dermatotoxicity, neurotoxicity, embryotoxicity and carcinogenicity) but as a first “crude contribution” to risk assessment.

2.2.“In vitro” models for metals in different toxicological areas

2.3. Metal dermatotoxicity

The skin is a target organ susceptible to the action of metal compounds absorbed locally and/ or systemically. Adverse reactions may be caused by both direct toxic effects and by the perturbation of natural levels required for homeostasis (e. g., cell- mediated immunity can be depressed by an excess or a deficiency of key metal species). Although skin is a generally efficient barrier to the penetration of metals and metal compounds, it can function as a reservoir or sink for some dermally contacting materials, and it also plays a significant role in the excretion of others (Hostynek et al., 1993). For example, in the case of iron, skin is the only significant elimination pathway once the metal is absorbed; indeed, conditions causing severe chronic epidermal desquamation can result in iron deficiency. The skin is clearly an important organ in the clinical manifestation of allergy (Epstein et al., 1963). Although the skin is an excellent barrier to the passive penetration of allergens, it also actively processes invading haptens through the Langerhans cells. Thus, the skin is a main organ expressing the state of hypersensitivity. Although immunogenic metal

compounds may cause contact dermatitis as the main reaction, they also have the potential to induce type I, immediate sensitisation (Turk et al., 1987). The percutaneous absorption of metal compounds appears to be influenced by multiple factors including their molecular size, charge, electropositivity, chemical reactivity, and oxidation state. The nature of the metal compound (salt vs, organometallic) also affects profoundly the rate of skin penetration. Thus, a simple algorithm for predictive purposes is not yet on hand, and only limited, quantitative penetration data are available for interpolation and extrapolation. In this context, metal compounds are able to penetrate the stratum corneum with a different penetration rates (Hostynek et al., 1993). Transepidermal (primarily intercellular) and transappendageal permeation pathways have been also identified for metal compounds. Penetration via hair follicles, sebaceous glands, and sweat glands has been established. However, “a priori” prediction of the major route of transport for any particular compound is not presently possible. Concerning lipophilic organometallic compounds, it has been shown that they are more easily absorbed across the skin, in relation to metal coordination complexes and electrolytes (Hostynek et al., 1993). Metal- ion size and charge also influence penetration. Transition metals in groups Vb, Vib, and VIII (e. g., Cr, Cd, Hg) have a particular capacity to coordinate with nucleophilic S-, N-, and O- containing functional groups, which are abundantly found in epidermal proteins. Certain metals, on contacting the skin in their elemental state (e. g., Pb, Ni, Cr, Cu), are oxidised by fatty acids present on the skin surface and then penetrate the stratum corneum as the derivative salts (Schwarz et al., 1959). Several physicochemical factors, affect penetration route and pathway of metal compounds in skin: molecular size, electropositivity, oxidation state, chemical reactivity, chemical nature (including lipophilicity), concentration, pH, and water and oil solubilities. In terms of biological functions, metal compound penetration is influenced by: (a) the role of the metal in homeostasis, (b) the formation and/ or existence

of a reservoir, (c) the presence of specific sequestrants (e. g. metallothioneins), and (d) metabolism (oxidation/ reduction).

Keratinocyte cell cultures seem a suitable “in vitro” model to study metal dermatotoxicity. For many years toxicological studies in animals have been carried out in order to assess the risk of dermal exposure to metals. Direct effects on the skin include inflammation, oedema, epidermal hyperplasia, and induction of skin tumors. In this context, while toxicological research involving living animals is continuing, the importance of complementary studies by cell cultures is becoming increasing. Epidermal keratinocytes (Holtbrook and Hennings, 1983) or “in vitro” systems which include dermal components (Bell et al., 1983) are caluable “in vitro” models in understanding the response of skin cells to external stimuli. Many reports of the successful organ, explant, and cell cultures of epidermal keratinocytes from various species have appeared in the past 25 years (Holtbrook and Hennings, 1983). The extent of maintenance of the differentiated phenotype varies with culture techniques, but final verification by grafting to the backs of athymic mice (Strickland et al., 1988) has been reported with each type of culture.

Recently, the development and validation of “in vitro” toxicity testing methods for regulatory purposes have found an important place in the European legislation. These methods, validated under the umbrella of ECVAM (3T3 Neutral Red Uptake photoxicity test, Epi Skin skin corrosivity test, rat TER skin corrosivity test and EpiDerm corrosivity test), represent alternatives non- animal test methods of relevant interest to replace animal testing in cosmetic industry, a field that involves also the inorganic aspect which has been , however, neglected until now (Table 2.1).

Table 2.1 summarises some chemicals used in cosmetology to which man is currently exposed.

Table 2.1- Metals used in cosmetology

Metal	Metal- based additives (FDA, Federal Drug Administration)
Ag	Powder
Al	Powder
Bi	Citrate/ oxychloride
Co	Cr- Co- Al hydroxide
Cr	Cr- Co- Alh, Cr oxide/ hydroxide (green)
Cu	Cu- Na_2EDTA/ Chlorophillin complex
Fe	Oxide/ ferrocyanide
Mn	Violet
Pb	Acetate
Sn	Powder
Sr	Chloride/ acetate/ lactate
Ti	Dioxide
Zn	Oxide

2.3.1. HaCaT cell line

HaCaT (Human adult, low Ca^{2+}, high Temperature) is the abbreviation that defines the origin of these keratinocytes. They originate from the skin of a human adult isolated with low concentration of Ca^{2+}, at high temperature (Boukamp et al., 1988). Although this established cell line maintains the characteristic of epidermal differentiation, is immortal (> 140 passages) and has a transformed “in vitro” phenotype, it remains nontumorigenic. In addition, HaCaT cells from passage 80 or higher show a comparable induction of suprabasal keratins in response to vitamin A depletion in the culture medium. In this way, the HaCaT cell line is closely approximated to normal keratinocytes, and thus offers a

suitable model to study regulatory mechanisms in the process of differentiation of human epidermal cells. Despite the altered and unlimited growth potential, HaCaT cells, similar to normal keratinocytes, reform and orderly structured and differentiated epidermal tissue when transplanted onto nude mice. The specific keratins and other markers are regularly expressed. The karyotype is aneuploid with chromosomal stabilised markers that indicate the monoclonal origin of this cell line (Jefrey et al., 1985).

2.4. Metal neurotoxicity

The nervous system is one of the most complex organs in terms of structure and function and it is particularly sensitive to toxic insult as nerve cells are often not capable of regenerating after exposure to neurotoxic insult (Costa, 1998).

In particular, interest in metal neurotoxicology has increased in response to the growing recognition that:

- Some environmentally and occupationally metals are known to be neurotoxic and may have a role in neuro degeneration and ageing, while even more are of suspected or unknown neurotoxicity.
- Little is known about the mechanisms involved in metal neurotoxicity.
- Many different chemical forms of metals have never been adequately tested for neurotoxic potential.
- There is a need to test metal compounds for neurotoxic potential (Atterwill et al., 1991; Walum et al., 1992; Binding et al., 1996; MacPhail et al., 1997).

The complexicity, and diversity of the nervous system has precluded the rapid development of “in vitro” alternatives for metal neurotoxicity testing (Williams et al., 1994). In fact, the use of “in vitro” models has been mostly used for mechanistic studies in neurobiology and neurotoxicology, (Atterwill et al., 1994; Costa, 1998).

Systems involving primary cultures offer the possibility of studying the neurotoxic effects trace metals on different cell types, like neurons, astrocytes and oligodendrocytes, but cannot provide the cytoarchitecture of the nervous system and neuronal circuitry of the specific brain area (Costa, 1998). In general, primary cultures express the more- normal properties of neurons or glial cells relative to their respective, transformed counterparts. However, they contain multiple cell types and they may require longer differentiation times. Moreover, primary cultures require more- complex nutrient media for survival and they need to grow on a substratum to support attachment, and this can lead to experimental variability (Veronesi et al., 1997). It has been suggested, for example, that primary astrocyte- enriched cultures may have a potentially valuable role in “in vitro” neurotoxicology (Cookson and Pentreath, 1994). Useful hybrid cell lines can result from a fusion of primary cells with, for example, neuroblastoma cell lines (Veronesi et al., 1997).

From the point of view of reducing the numbers of animals, cell lines represent the best alternative, as this model is the only one not using cells or tissues directly derived from animals (Costa, 1998). Representatives of neurons (neuroblastomas), and glia (oligodendrocytes, schwannoma, astrocytomas) from various species are commercially available (Veronesi, 1992). In addition to cell lines derived from tumors, more recently, the techniques of genetic engineering have resulted in the production of cell lines with specific desired characteristics. This has been accomplished by the insertion of desired genes into preexisting immortal cell lines or, alternatively, by immortalising primary cells. In the nervous system, these techniques have produced immortal cell lines with neuronal and glial properties (Geller et al., 1991). The advantages of using cell lines are:

- commercial availability, self- propagation;
- growth in chemically- defined media;

- the ability to maintain frozen cell stocks that can be used for the inter- laboratory validation of methods;
- the large amount of information on their physiology and biochemical composition; and
- the reproducibility of results is often better in cell lines than in primary cell cultures (Veronesi et al., 1997; Costa, 1998).

For example the human neuroblastoma derived cell line, SH- SY5Y, has been suggested for use as an “in vitro” model in the pre- clinical screening of potentially neurotoxic agents and in studying the pathophysiology of drug- induced neurotoxicity .

The disadvantage of cell lines, besides those cited also for primary cells, is that they are transformed and lack many of the specific characteristics of their cells of origin. Chemicals which disrupt the integrity of the blood- brain barrier (BBB) and gain access to the neuropile can be especially devastating (Veronesi, 1996). “In vitro” systems that could mimic the BBB have been characterised, for example consisting of co- cultures of glial cells and endothelial cells (Balls and Walum, 1999; Stannes et al., 1996; Veronesi, 1996). Their potential value for mechanistic neurotoxicological studies is great (Pentreath, 1999), and they are being tested for their suitability for detecting neurotoxins.

Table 2.2 shows endpoints used in “in vitro” neurotoxicity testing and the corresponding measurements of the endpoints. If nervous system- specific endpoints are affected at concentrations lower than those producing cytotoxic effects, a chemical can be considered to be a potential neurotoxin (Atterwill et al., 1994).

Table 2.2- Some endpoints used in "in vitro" neurotoxicology. Modified from Atterwill et al. (1994).

Endpoints	Measurements of endpoints
General endpoints	
Necrosis	Neutral red uptake, MTT reduction ,fluorescein diacetate hydrolysis,lactate dehydrogenase leakage, ethidium bromide
Apoptosis	ELISA[a] for determination of nucleosomes DNA laddering, TUNEL[d] , flow cytometrical/ morphological evaluation after dying with fluorescent dyes
Proliferation	Cell counting, [H^3]- thymidine incorporation, flow cytometry
Differentiation	
Glia	Glial fibrillary acidic protein (GFP), monoamine oxidase B, myelin basic protein
Neurons	Neurite- like outgrowth, neurofilament protein (NFP), transmitter metabolism, uptake and content, microtubule- associated proteins
Cell homeostasis	Voltage sensitive and ion- sensitive fluorescent dyes
Specific endpoints	
Receptors	Radioligand binding
Ionotropic	Electrphysiology, dye measurements (Fluo- 3, Fura- 2)
Metabotropic	Cyclin nucleotides (RIA[b]),
	Inositol phosphates (radio- labelled or mass measurement),
	Intracellular pH (cytosensor)
Ion channels	Electrophysiology, ion fluxes ^{86}Rb, ^{22}Na, ^{45}Ca, ^{36}Cl), dye measurements
Signal transduction	Protein phosphorilation, [^{32}P]ATP incorporation, blotting
Enzymes	Acetylcholinesterase, choline acetyltransferase, monoamine oxidase, neurotoxic esterase (NTE)
Uptake systems	Radio- labelled ligand uptake
Release	Radio- labelled tracers, electrocapacitance, endogenous release (HPLC-ECD[c],RIA[b], bioassays for cytokines)
Energy metabolism	ATP levels (luciferin/ luciferase assay, HPLC)

a= enzyme- linked immunoabsorbent assay

b= radioimmunoassay

c= high perfprmance liquid chromatography- alectrochemical detection

d= terminal deoxyuridine nucleotide and labelling

Before “in vitro” can begin to gain industrial and regulatory acceptance, they have to validated in order to correlate “in vitro” results with neurotoxicological responses in whole humans or animals (Sobotka et al., 1996). At present, no validation has been done in the field of neurotoxicity testing. Only studies have been performed in which metals were tested and different testing bacteries were used (Costa, 1998).

At present, “in vitro” studies on neurotoxic effects of metals included different “in vitro” systems and are mainly limited to Pb, Cd and Mn. It is known that some trace metals induce neurotoxic effects due to disruption of ionic mechanisms involved in neurotransmission, including calcium translocation across the neuronal membrane (Bressler, 1991). Studies on the interaction of lead with voltage- gated calcium channels have uniformly reported a reduction in current amplitudes following acute lead exposure in invertebrate neurons (Audesirk, 1993; Buesselberg, 1993), mouse and human neuroblastoma cells (Audesirk, 1991; Oortgiesen, 1993; Reuveny, 1991; Vijverberg, 1994 and Evans, 1991)., rat hippocampal neurons (Audesirk, 1993), and rat dorsal root ganglion neurons (Buesselberg, 1993, 1994). The effects of lead in these widely different experimental models were qualitatively similar, although cell types were unequally sensitive to trace metals action (Hegg et al., 1996).

The mechanisms of metal neurotoxicity have poorly been investigated and limited to fewmetals. Cadmium and methyl mercury disrupt neuronal function by altering the function of multiple cellular proteins and calcium homeostatic mechanisms, including voltage- gated calcium channel function (Shafer, 1998). Voltage- gated calcium channels have been shown to be sensitive targets for trace metals. Cadmium (Chow, 1991; Thevenod and Jones, 1992) and methyl mercury (Shafer and Atchison, 1991a; Leonhardt et al., 1984; Sirois and Atchison, 1997) block N- and L- type calcium channel currents with IC_{50} values < 10 μM (total metal). Cadmium permeability through calcium channels is

very poor relative to that of calcium, and cadmium is an effective blocker of calcium uptake (Lansman et al., 1986; Tsien et al., 1987). Despite this, the amount of cadmium entering through voltage- sensitive calcium channels can be significant in cells with high calcium channel activity, and organic calcium channel blockers protect such cells from the toxic effects of cadmium (Hinkle et al., 1987; Flanagan and Friedman, 1991; Blazka and Shaikh, 1991; Borowitz and McMaughlin, 1992).

Manganese is another neurotoxic element able to promote neurite outgrowth in rat pheochromocytoma (PC12) cells (Walowitz and Roth, 1999). Classic neurotoxic symptoms from overexposure to manganese (Mn) consist of psychomotor excitement, irritability, and compulsive behaviour in the early stages of the disorder to more pronounced and severe extra pyramidal symptoms upon prolonged exposure (Huang et al., 1993; Shukla and Singhal, 1984; Rodier, 1955). Chronic exposure manifests itself in neuronal degeneration characterised by dyskinesias resembling Parkinson's disease including tremors and difficulty in walking. In contrast (Shukla , 1984; Chandra and Shukla, 1981; Chandra et al., 1979), acute exposure to manganese paradoxically causes hyperactivity accompanied by elevated brain levels of catecholamines and their metabolites. Manganese intoxication in humans is usually identified with prolonged occupational exposure to dangerously high levels of this metal. However, several recent articles (Pomier- Layragues et al., 1995; Krieger et al., 1995) have indicate that patients with chronic liver failure may also be at particular risk to manganese poisoning. These latter studies suggest that some of the behavioural deficits and neurodegenerative feature observed in patients with liver failure are typical of patients with manganese neurotoxicity (Walowitz and Roth, 1999).

Table 2.3 summarises the neurotoxicity induced in humans by trace metals to which man is currently exposed.

Table 2.3- Neurotoxicity of metals observed in humans

Metal	Type of exposure		
	Environmental	Occupational	Iatrogenic
Au			(+)
Al			(+)
As	(+)	(+)	(+)
B		(+)	
Bi			(+)
Hg	(+)	(+)	
Mn		(+)	
Pb	(+)	(+)	
Pt			(+)
Sn		(+)	(+)
Te		(+)	
Tl	(+)	(+)	(+)

2.4.1. PC12 cell line

The rat pheochromocytoma cell line, PC12, was originally cloned from a transplantable rat pheochromocytoma (Green and Tischler, 1976). PC 12 cells exhibit the phenotypic properties associated with pheochromocytomas and their non- neoplastic counterparts, adrenal chromaffin cells. The cells synthetise, store and can release catecholamines (principally dopamine and norepinephrine; these cells do not express detectable amounts of epinephrine), and respond reversibly to nerve growth factor (NGF) by induction of the neuronal phenotype and the acquisition of a number of properties characteristic of sympathetic neurons. For instance, the NGF- treated cells cease proliferation, extended

long, branching neuronal- like processes, become electrically excitable, express new receptor proteins, and show a number of changes in composition associated with enhanced neuronal differentiation. PC 12 cells display a polygonal morphology, are poorly adherent to plastic, grow in small clusters, and display a doubling time of 92h. The PC 12 cell line is frequently used in neurobiology as it is relatively stable, homogeneous, has a high degree of differentiation, shows a vigorous response to NGF, and has the potential for genetic manipulation (Greene et. Al., 1988). The disadvantage of this cell line is that it lacks functional N- methyl- D- aspartate (NMDA) channels (Rossi et al., 1997). PC12- p53 engineer modified cell line contain the gene that codes for protein p53. p53 is a protein with a wide array of physiological and biological function, including safeguarding the integrity of the genome, and in cell cycle regulation, apoptosis, differentiation and angiogenesis (Stingele, 2000). p53 is expressed at very low levels in normal cells. It has a half- life of only about 20- 30 minutes, but accumulates (Huges et al., 1997) in response to DNA damaging agents such as UV- irradiation or γ- irradiation and genotoxic compounds (Kastan et al., 1991; Maltzman and Czyzyk, 1984 and Johnson et al., 1998), or physiological stress conditions, such as hypoxia and heat (Graeber et al., 1994; Ohnishi et al., 1996).

Interestingly, the modification of the growth medium influences the expression of p53 gene. Metal solutions in a medium tetracycline free (Tet- on) induce p53 expression, while a medium with addition of tetracycline (Tet- off) leads to a lack of p53 expression.

2.5. Metal embryotoxicity

Very little is known about the role of metals in embryonic development and on their teratogenic effects. Methyl mercury (MeHg) is certainly one of the more investigated by “in vivo” and “in vitro” methods metal ocmpound. It crosses the mammalian placenta and has been shown to reach a 30% higher concentration in foetal erythrocytes than in maternal red blood cells (Suzuki et al., 1967). In this study on mice, the reported average foetal erythrocyte levels were 0.115 μg/ gram red blood cell (RBC), while comparable average maternal RBC levels were 0.0086 μg. Newborn plasma levels were lower than red cell levels by a factor of 100, suggesting that methyl mercury may selectively bind to foetal RBC’s. but in this study only 14 mother and newborn pairs were examined, limiting the interpretation of this animal data. For mice, placental transfer and increased foetal erythrocyte binding of mercury suggest that the greater risk of mercury to the foetal nervous system may be due, in part, to foetal mercury “trapping”. There are no comparable human data available which simultaneously measure maternal and foetal mercury levels.

Methyl mercury has been found giving reproductive effects (Burbacher et al., 1984) when animals were exposed at levels that do not produce overt toxicity. It has been shown that reproductive dysfunction is one of the earliest effects of MeHg administration in adult females. Increased blood Hg concentrations were associated with decreased fertility and increased early spontaneous abortion (Burbacher et al., 1984). The embryopathic effects of congenital MeHg administration include alterations in behaviour (Spyker et al., 1972) and morphology (Fuyuta et al., 1978) as well as a reduction in the size of the offspring (Mottet, 1974; Fuyuta et al., 1978). Behavioural alterations which included motor in coordination and increased activity and decreased offspring size were demonstrated in prenatally exposed mice at doses of MeHg which did not affect maternal morphology or behaviour (Burbacher et al., 1984).

Different experimental studies have been reported on the embryotoxic and teratogenic effects of MeHg in rats (Ramel, 1967; Matsumoto et al., 1967; Moriyama, 1967; Nonaka, 1969; Inoue et al., 1972; Casterline and Williams, 1972; Nolen et al., 1972a, b; Khera and Tabacova, 1973; Scharpf et al., 1973; Ware et al., 1974; Mottet, 1974; Chang and Sprecher, 1976), mice (Spyker and Smithberg, 1972; Khera and Tabacova, 1973; Su and Okita, 1976a, b) cats (Moriyama, 1967; Khera, 1973) and hamsters (Harris et al., 1972). Interestingly, in general selenium compounds seem to have a protective effect on the toxicity of MeHg in rats (Iwata et al., 1973; Johnson and Pond, 1974; Potter and Matrone, 1974; Stilling et al., 1974; Ueda et al., 1975; Ohi et al., 1975a, b, 1976) and in Japanese quail (Ganther et al., 1972; Stoewsand et al., 1974)., while an excess of selenium has an adverse effect on reproduction in some experimental animals (Rosenfeld and Beath, 1954; Schroeder and Mitchner, 1971a). However, the effects of selenium on the embryotoxicity and teratogenicity of MeHg have not been investigated.

Table 2.4 summarises the reproductive toxicity of some metals and their site of damage.

Table 2.4- Reproductive toxicity of metals.

Metal	Site of damage
Cd	Non spermatogenic tissue
Cr	Interstitial tissue
Ni	Spermatozoa
Pb	Chromatin of spermatozoa; seminal vesicles; prostate; endothelial cells of testis

2.5.1. Embryonic stem cells, D3

The ES cell line ES- D3 was derived from eight 129/ Sv+/ + 4- day blastocysts, day of plug detection being set at 1 day of embryonic development (Doetschman et al., 1985).

Blastocyst- derived embryonic stem (ES) cells are established “in vitro” from substrate-attached blastocysts without passage of the cells through tumors. They are maintained in an undifferentiated pluripotent state by culturing on an embryonic fibroblast feeder layer and spontaneously differentiate in the absence of feeder layer cells (Doetschman et al., 1985). All blastocyst- derived ES cell lines so far described spontaneously differentiate and form cystic embryoid bodies (Evans and Kaufman, 1981; Martin, 1981; Robertson et al., 1983). The degree to which organised development similar to that of the embryo occurs within them, however, has not been described. The investigation reported here has done this by analysing the most advanced embryonic- like structures developed by a blastocyst- derived cell line, ES- D3. It has compared the extent of this development, as well as that of several other ES cell lines from 129 and C57 mouse strains, to the postimplantation embryo. It is shown that the blastocyst- derived cells can differentiate at a remarkably high frequency to form heart and blood cell- containing cystic structures similar to the visceral yolk sac of the embryo (Doetschman et al., 1985).

It has been shown that ES cells can be genetically manipulated to generate transgenic or “knockout” mice (Thomas and Capecchi, 1987), and “in vitro” cell culture models were established to study myogenesis, angiogenesis, hematopoiesis, neurogenesis and cardiogenesis in the mouse (Wiles and Keller, 1991; Wobus et al., 1991; Heuer et al., 1994; Maltsev et al., 1994; Rohwedel et al., 1994; Struebing et al., 1995; Kolossov et al., 1998).

The differentiation of ES cells into cardiac cells (Doetschman et al., 1985) has been used in investigations on prenatal pharmacology, electrophysiology and molecular genetics

(Wobus et al., 1991; Maltsev et al., 1993; Metzger et al., 1996; Kolossov et al., 1998, Scholz et al., 1999).

2.6. Metal carcinogenicity

1. The carcinogenic potential of trace metals is a matter of European regulation. Considering European regulations there is a Directive 87/302/EEC that establish guide lines for “in vitro” morphological transformation in relation to the classification, preparation and marking of dangerous substances (Yamasaki, 1995; O. J. EEC, 1988). In this context, some metal compounds show a carcinogenic activity classified as “moderate vs strong” in laboratory animals (Gold, 1984- 1992; Sunderman et al., 1987; Sunderman, 1971; Sunderman, 1975; Sunderman, 1977; Sunderman, 1984). It is known that some of these compounds are linked to human carcinogenicity (Lee, 1965; Furst, 1977; IARC, 1980; Woo et al., 1981; Leonard et al., 1984; Rall, 1991; IARC, 1973; IARC, 1993; NTP, 1994; Landsdown, 1995). In fact IARC (International Agency for Research on Cancer, Lyon) established an evaluation grade of the carcinogenic potential of some metal compounds in human (Boffetta, 1992) (Tables 2.5, 2.6)

Table 2.5- IARC (International Agency for Research on Cancer, Lyon) criteria to establish an evaluation grade of the carcinogenic potential of metal compounds.

Group	Carcinogenic potential	N° of evaluated chemicals
1	Carcinogenic for humans	57
2A	Probable human carcinogen	50
2B	Possible human carcinogen	191
3	Non human carcinogen	443

Table 2.6- IARC classification of the carcinogenic potential of metals and their compounds (Boffetta, 1992).

Metal	Group	Evidence	
		Human	Animal
As	1	Sufficient	Limited
Be	1	Limited	Sufficient
Cd	1	Limited	Sufficient
Co	2B	Not ad equated	
Cr (VI)	1	Sufficient	Sufficient
Cu	3	Absent	Limited
Ferric oxide	3	Not ad equated	Sufficient
Occupational exposures to Fe	1		
Inorganic fluorides used in drinking water	3	Not ad equated	Not ad equated
Hg	Evidence in animals		
Mn	3	Not ad equated	Not ad equated
Ni	Evidences in human and animals		
Pb and its compounds	2B	Limited	Limited
Cis PDD Pt (II)	2A	Not ad equated	Sufficient
Sb trioxide	2B	Not ad equated	Sufficient
Sb trisulfoxide	3	Not ad equated	Limited
Se	3	Not ad equated	Not ad equated
Sn	No data in humans		
Ti dioxide	3		Limited
V	No data in humans		
Zn	No data in humans		

Concerning the possible mechanisms relative to transforming action induced by metal compounds, it is known that, being cancer a multi- stadium process, the toxic action of metals can be the result of interference or damage at different step of the process. For example chromosomic damage, DNA repair, inhibition of DNA repair, DNA linkage, wrong synthesis of DNA, stress proteins activation, inhibition of cellular communication. All these processes can occur dependently from the oxidation state of the different metal compounds (Mazzotti, 1999).

Table 2.7 shows studies relative to the carcinogenic potential of metal compounds in BALB/3T3 systems, the cellular model used in the present work.

Table 2.7- Metal compounds tested by morphological transformation assay using BALB/3T3 cell line[a].

Metal	Compound	Transformation	Bibliography
As	NaAsO2	+	Bertolero et al. (1987).
	Na2HasO4	+	Bertolero et al. (1987).
	(CH3)2As(O)OH	-	Sabbioni (personal communication).
	CH3Has(O)OH	-	
	(CH3)3AsCH2C(O)OH	-	Sabbioni et al. (1991).
Cr	CaCrO4	+	Dunkel et al. (1981).
Ti	(C5H5)2TiCl2	+	Dunkel et al. (1981).
V	NH4VO3	+	Sabbioni et al. (1991); Sabbioni et al. (1993).
	VOSO4	-	Sabbioni et al. (1991); Sabbioni et al. (1993).
	Na3VO4	+	Sheu et al. (1992).

a: += transformation activity; +/-= weak transformation activity; -= no observed transformation activity.

2.6.1. BALB/3T3 cell line

BALB/3T3 cell line derives from subclones (Saaronson et al. (1968)) of the A31 clone, originally isolated from embryonic mouse cells. (Saffiotti et al., 1984). The BALB/3T3 Cl A31- 1- 1 cell line of mesenchimal origin was chosen since it is one of the recommended system to study basic aspects of the response of mammalian cells to carcinogens and since it is one of the most valuable short term tests for bioassays permitting both a qualitative as well as quantitative dose- dependent response to carcinogens including trace metals (Bertolero et al., 1987).

This cell line is heterogeneous and the different subclones differ from each other by the different way of spontaneous transformation and different sensibility to induce transformation. The clone has been chosen because shows a spontaneous incidence of transformation included between those two values 10^{-5} and 10^{-6}. The transformation frequency increases of some order of magnitude with known carcinogens (es. to $>10^{-2}$ for benzo(α)pyrene) (Saffiotti et al., 1984). Is also known that the spontaneous transformation frequency is established for those cells with a low passage (subculture, cells are transferred from initial flask to another) and becomes higher with the age of the cell line. To interpret the results is important to remember that cells of this line are not comparable with normal cells because these cells have acquired the phenotypic characteristics of transformed cells like immortalisation, aneuploid number of chromosomes and high plating efficiency (50-60%). In the meantime they maintain characteristics of normal cells like contact inhibition (if put in culture they are able to form a monolayer), like the dependence of the growth from the anchorage and the non tumorigenicity. They have also conserved the possibility to be furtherly transformed in “in vitro” cultures (Saffiotti et al. 1989). Clone A31- 1- 1 has a number of chromosomes subtetraploids. The karyotypic analysis revealed the 98 % of the

metaphases contained between 70 to 80 chromosomes. This number is increasing (100-140) when the cells are incubated with a carcinogenic substance (Saffiotti et al. 1984).

A quantitative dose- response relationship between carcinogen concentration and morphological transformation rate is obtainable with these cell lines while untreated controls or cultures treated with noncarcinogens do not show transformation or produce tumors. Transformation is calculated on the basis of either transformed colonies or transformed foci on a background of normal cells. The transformation foci consist of cell colonies that are able to grow in non organised and invasive layers in comparison to the surrounding monolayer. The foci are of three types:

Type I: focus of lightly group together cells.

Type II: focus made of mass of cells grown in multilayer.

Type III: focus made of cells strictly linked one over another.

The peripheral part of the colony is usually characterised by criss- cross cells grown in a disorganised and invasive way.

In the evaluation of the transforming potential effect, only types 3 foci are counted as malignant transformations. An elevated percentage (85 %) of these foci are able to origin neoplasia when injected in nude mice (Saffiotti et al. 1984).

Although BALB/3T3 system was reported to be susceptible to transformation by a range of different organic chemical carcinogens, data on metal- induced transformation were lacking and only recently a systematic study was carried out on several metal compounds.

2.7. Selected Metals

2.7.1. Platinum as new potential environmental pollutant

Platinum, palladium and rhodium are required for “three- way catalyst” of cars to control the three noxious exhaust car emissions, namely carbon monoxide, hydrocarbons and nitrogen oxides (Damiano, 1990). Since automobile catalysts are mobile sources of platinum, palladium and rhodium, (some loss of the elements can be released into the environment due to mechanical and thermal impact) emissions from these car catalysts suggest concern the platinum could represent a “new” potential environmental pollutant.

2.7.2. Environmental levels and levels in diet, biological fluids and human tissues

Platinum is a metal present on earth crust at parts for billion level. The more common oxidation states are (+2) and (+4). The known deposits are localised principally in Siberia, in South Africa, (the major Pt producer), and in Canada (Loebenstein, 1988).

Data on platinum emissions from automobile catalysts are very limited. Engine test stand experiments were carried out in Germany (Fraunhofer Institute of Toxicology and Aerosols Research, Hannover) in 1990 as part of a programme of the Ministry of Research and Technology for assessing the health risk of this man- made environmental source. The results showed platinum emissions from few to some tens ng m^{-3} (Hill et al. 1977 and H. P. Koenig et al., 1989) depending on the temperature. It has been suggested that platinum is released as metal or oxide (an important aspect for toxicological considerations).

Air concentrations of platinum were calculated between 0.005 to 1.3 ng Pt m^{-3} near and on roads, and 0.9 ng m^{-3} in tunnels (Tab. 2.8).

Table 2.8- Quantity of Pt present in air of different sites.

Site of exposure	Pt concentration (ng/m^3)
Personal garage	0.8-6.7
Silos	0.4-5.6
Galleries	0.4-0.9
On roads	0.07-0.16
Near roads	0.004-0.13

(Rosner et al., 1990).

Studies in Germany gave workplace concentrations of 0.08-0.01 μg Pt m^{-3} for soluble Pt compounds.

There are little information concerning Pt level in water. Typical values are: 0.1-0.25 ng Pt L^{-1} in oceanic water and 110ngPt L^{-1} in mineral water.

Few data on Pt levels in human tissues and biological fluids are reported in literature (Pietra et al., 1994). Vaughan and Florence (1992) studied Pt in Australian diet. The daily ingestion don't exceed 1.5 μg Pt/person. The intestinal absorption of Pt, estimated time ago was near 42 %, a value which today is not realistic. The absorption of $PtCl_4$ is much lower (less than 1%) (Filov, 1977).

Some scientific reports show that Pt is increasing in blood/ urine of general population. Anyway this is not fully demonstrated due to technical difficulties in establishing the "normal" reference values of Pt in human fluids and tissues. In the context of the EURO TERVIHT project (Sabbioni, 1992) (Trace Element Reference Values In Human Tissues) 40- 50 ng L^{-1} of platinum were found in blood/ urine of people living in Northern Italy (Unpublished results).

It has been suggested that Pt would be mobile in the different compartments of the ecosystem. This is may be due methylation by vitamin B12 (Wood, 1974).

2.7.3. Toxicological/ carcinogenic effects and estimation of health risk

The main health hazard of Pt compounds in asthma and allergy as observed in occupational workers. However, the data are extremely limited to believe that Pt emissions from car catalyst can induce hypersensitivity and so, at present, any attempt to link the allergenic effect of Pt salts with Pt mobilised from car catalyst is not based on a scientific evidence. Until recently, the exposure to Pt salts was confined to occupational settings (Pt refinery and catalyst production plants). In this case the main health hazard is asthma (Linnet, 1987). These allergic symptoms are characteristic of a pathology described since 1977 as "Platinosis" (Parrot et al., 1969) (Tab. 2.9).

Table 2.9- Reports on Pt allergy.

Date	Discovers	References
1911	First report on Pt allergies in exposed workers.	Karasek, Karasek (1911)
1945	Clinic evaluation of Pt allergies in exposed workers.	D. Hunter et al. (1945)
1951	Born of the term "platinosis".	F. Roberts (1951)
1955	First contact sensibility report to Pt compounds.	C. Sheard (1955)
1972	Report concerning the damage on the skin and on the respiratory tract in exposed workers.	J. Pepys et al. (1972)
1979	Rast developed for Pt allergy.	O. Cromwell et al. (1979)
1980	Supervision of the exposed workers.	E. G. Huges. (1980)
1988	Report on Pt allergies.	R. Merget et al. (1988)
1990	Pt allergy on refinery workers.	U. Bolm-Aurdoff et al. (1990)

An incidence of asthmatic symptons of about 50 % at a workplace air concentration of 0.1 μg Pt m^{-3} has been estimated. This air concentration should be higher by a factor 1,000 compared to the concentration of platinum of 0.1 ng m^{-3} calculated at busy roadsides. In addition, the insoluble chemical form of Pt emitted from car catalysts may result in even much lower ambient air concentration of total Pt emitted. So, it is unlikely that platinum emitted from car catalysts causes asthma.

Platinum compounds are not allergenic “per se” because they have a low molecular weight. They would act as haptens able to bind carriers with a high molecular weight like blood proteins. Some studies demonstrate that Pt (II) is able to react with –SH groups of albumin (Trynda et al., 1994) which is the most abundant plasmatic protein and with human transfer proteins (Trynda et al., 1994).

An important aspect which is the subject of newspaper articles is the potential carcinogenic of Pt compounds. There is a lack of information to assess the carcinogenicity/ mutagenicity of Pt compounds. The alarmism which appeared on this subject (Pt emission from car catalysts could cause cancer) is completely unjustified. When we speak on carcinogenicity of metals we must take into account their chemical form, because it is known that different chemical species of an element can lead to completely different toxicological/ carcinogenic effects. Most of the small amounts of Pt emitted from car catalyst is presumably in form of metal or oxide. These chemical species must not be confused with the cis- Platinum which is used as an antitumor drug at pharmacological doses (Pooly and Lohman, 1980) (cis- Pt has a proper neoplastic activity and it is classified as “ probably carcinogenic to human, (category 2A0 by the International Agency of Research of Cancer (IARC)) (Boffetta, 1993). Thus, the extrapolation of the moderate neoplastic action of the Pt and cis- Pt to the

problem of Pt emitted in mostly insoluble metal (or oxide) chemical species from car catalyst has little sense and can be considered a speculation.

At present, it is considered that the health risk arising from the emission of Pt from car catalysts should be “low”. This conclusion, however, is based more on lack of available data rather than on a scientific evidence. So, research in this field is necessary and should include: a) analytical work (geochemical maps showing concentration of Pt in top soil and sediments; extent and chemical form of Pt released to the environment; establishment of current levels of Pt in body fluids and tissues of different subjects, e. g motorway maintenance workers, population living near motorways and general unexposed population) b) toxicological studies to set dose- effect relationships of Pt in experimental and human models which should also include studies at workplace as well as in “in vitro” cell culture models.

2.8. Methyl mercury, a well known poisoning

Organic mercury compounds have been characterised as hazardous, especially the alkylated compounds. Because of the alkylated mercury compounds’ long retention by the human body, because of their toxic effect on developing tissue and furthermore, because of their known propensity for central nervous system damage, alkylated mercury compounds pose particular and significant hazards for the fetus. Toxicologically and environmentally the most dangerous alkyl compound is methyl mercury (Grass, 1969).

Mercury and its congeners are extremely toxic substances which exist in several physical and chemical forms: inorganic mercury, organic mercury and mercury vapour. Sources of human exposure to mercury include seafood, seeds, foodstuffs, water and dental amalgam (Shenker et al., 2000).

2.8.1. Environmental exposure

Documented sources for mercury contamination of the environment are waste discharges of chlorine and caustic soda manufacturing plants, from mercury catalysts used in industry, from fungicides used in the pulp and paper industry, from pharmaceutical manufacturing by products, and from the burning of fossil fuels (Nelson, 1971). These industrial sources contribute over 70% of the mercury added annually to the American environment (Gold, 1971). Other miscellaneous sources include medical and scientific wastes, naturally occurring geological formations, and the processing of raw ores containing mercury. Evidence suggests that environmental mercury content is increasing due to man's activity. The mercury content of Greenland ice has more than doubled in the past 50 years (Weiss et al., 1971). Once inorganic mercury has been released into the environment, methylation occurs by bacterial action. This has been demonstrated in the sediment of waterways (Jensen, 1969) Methyl mercury is then available to ascend the food chain, reaching human consumption via edible fish, shellfish, or water fowl.

Health hazards posed by these sources are based mainly upon data collected following acute exposure to high levels of mercury as a result of occupational or industrial accidents (Shenker et al., 2000). Concern, however, has recently shifted to the potential hazards of chronic exposure to low levels of mercury and, in particular, the possibility of adverse effects on the human immune system. Chronic exposure to low concentrations of heavy metals, such as mercury, results in immune dysfunction (Clarkson, 1997; Pollard and Hultman, 1997; Zelikoff and Gardner, 1996). Since immunotoxic effects can lead to immunoregulatory deficits, mercury may then be capable of triggering immunologically mediated disease (such as autoimmunity) or promoting chronic infection (Silbergeld et al., 1998); moreover, there is the possibility that immune dysfunction could influence the development and progression of cancer (Shenker et al., 2000). For instance, there is

evidence that exposure to both organic and inorganic mercury results in immune activation leading to adverse outcomes related to allergy and autoimmune disease (Dieter et al., 1983; Lawrence, 1981; Thuvander et al., 1996; Warfvinge and Larsson, 1994). Additionally, mercurial species have been shown to be cytotoxic and to possibly contribute to chronic and/ or recurrent infection (Aten et al., 1995; DeFlora et al., 1994; Franchi et al., 1994; Koropatnick and Zalups, 1997; Langworth et al., 1993 Osorio et al., 1995; Steffensen et al., 1994). Another contributing factor to these discordant observations may relate to differences in pharmacokinetics of exposure (ingestion vs inhalation) and sources of cells (human vs animal species/ strain).

Several investigations have shown that mercuric compounds cause cytogenetic alterations following exposure “in vitro” or “in vivo” (DeFlora et al., 1994; Franchi et al, 1994; Betti et al., 1993).

It has been also seen that prolonged dietary exposure to high concentrations of methylmercury (MeHg) has been associated with numerous behavioural and morphologic aberrations (Fuyuta et al., 1978; Spyker et al., 1972). Studies of MeHg- exposed populations in Iraq (Amin- Zaki et al., 1976; Bakir et al., 1973), Minamata (Harada, 1968), and Nigata (Harada, 1968) have reported clinical symptoms such as ataxia, constriction of the visual field, and mental disturbance, as well as pathologic lesions of the cerebrum and cerebellum.

2.9. Arsenic, as environmental pollutant

Arsenic is an element widely distributed in the biosphere. Its natural redistribution, however, can be significantly changed by human activities causing environmental pollution. For this, it occupies a prominent place among the pollutant singled out for

priority attention in terms of legislative action in the European Communities (Berlin, 1981).

The use of arsenical pesticides, of coal for electrical energy production, of fertilisers for agricultural purposes and releases from non ferrous smelters as well as geothermal power plants contribute to environmental arsenic pollution so that human exposure to arsenic can occur via inhalation of industrial dust and via ingestion of contaminated water and food (Sabbioni et al., 1985b).

Cases of environmental exposure to high levels of As are related to the ingestion of the metal geochemically from reached drinking water such as in the region of Cordoba (Argentina), in Taiwan and Antofagasta (Chile). Dramatic health effects were observed including high mortality from skin cancer (Pearce, 1993).

Possible routes of exposure to As for general population include air, food, water and smoke. The amount of As ingested daily by humans via food is greatly influenced by the content of seafood in the diet which is probably responsible of the large variations between few tens to some hundredth micrograms reported in literature. Smoke contributes significantly to the daily exposure (10- 30μg/day are inhaled by an average smoker). The daily exposure from water ordinarily will not exceed 5μg/day while that from air is less than 1μg/day (IPCS, Arsenic, 1983).

The knowledge of the chemical species of As in the possible routes of exposure for general population is particularly important in order to address correctly the biochemical research on laboratory animals. Table 2.10 summarises some chemical forms of As identified in air, water and sea food to which man is currently exposed.

Table 2.10- Chemical species of arsenic in the environment to which humans are exposed[a]

Compartment	Source of exposure	Identified chemical form
Air	Non- ferrous smelter	As_2O_3
	Rain (urbanised area)	As (III) (35 %)
	Smelteries	As_2O_3, As_2S_3
Water	Natural water	Inorganic; mainly As (III)
		Inorganic; mainly As (V)
		Organic; methylarsonic and dimethylarsinic
	Sea water	Inorganic; mainly (As (V), methylated forms
	Wine	Inorganic; mainly As (III)
Food	Sea- food	Organic; methylarsonate, dimethylarsinate; arseno sugars; arsenobetaine; arsenocholine; fat soluble arsenic compounds.

a: generalised from Sabbioni et al., 1985b.

2.9.1. Toxicological/ carcinogenic effects

The toxicological effects of inorganic arsenic are generally related to its oxidation state, trivalent arsenite being more toxic than pentavalent arsenate. Studies on laboratory animals showed dose- dependent retention of arsenite, implying that the higher toxicity of trivalent arsenic may related to its higher affinity for cellular constituents.

In addition, certain fish and crustacea contain high concentrations of arsenic, sometimes as much as 100 mg As/kg (Lunde, 1973). Although As compounds present in fish, mainly organo- arsenic compounds, arsenobetaine and arsenocholine are much less toxic than inorganic As species, their metabolism and possible adverse effects in mammals are

largely unknown. Since the toxicological differences between tri- and pentavalent As could be explained by differences in the chemical properties and hence biochemical behaviour, the study of the biochemical mechanisms for the toxic effects of As requires the knowledge of the interaction of As from different chemical forms with intracellular constituents as well as the biotransformations occurring in the body.Exposure of humans to inorganic trivalent arsenic leads to skin cancer (Pearce, 1993).

2.9.2. Biotransformations

Oxidation and reduction of inorganic arsenic have been observed in mice and rabbits (Bencko et al., 1976). Methylated forms of arsenic have been found in the urine of many animal species as well as in humans (Crecelius, 1977). It has been suggested that the elimination and the detoxification of adsorbed inorganic arsenic could be closely related to the methylation process. Therefore, the different retention of arsenic observed in different animal species could be due to differences in the methylation rate in tissues. On the other hand, arsenite is known to be a potent inhibitor of enzymatic activity, reacting strongly with sulphydrilic groups of the proteins (Vallee, 1960), while arsenate interferes with phosphorilation in the mitochondria due to its chemical similarity with phosphate (Fouler et al., 1977). The biotransformation and the tissue binding of arsenic in four species of laboratory animals showed that the interaction of arsenic with intracellular components varies according to the animal species depending from the biotransformation of inorganic arsenic to dimethylarsenic acid.

Mice, showing a very rapid methylation of arsenic in tissues, have the higher urinary excretion and the lower binding of arsenic in tissues Rat had the lowest urinary excretion and the highest retention in the body of the animal, particularly in the blood. (Vahter and Marafante, 1988).

In rats the low rate of methylation and excretion of arsenic seems mainly due to the peculiarly high affinity of the haemoglobin of this species for the arsenic. In rabbits a fairy weak interaction of arsenic with subcellular components was observed. There was a continuous excretion of As mainly in form of DMA related to a simultaneous decrease of protein- bound arsenic in tissues (Vahter and Marafante, 1988).

In none of these animal species we found the monomethylated form of arsenic, monomethylarsenic acid, which is present in human urine. Although this result seems to confirm that no perfect animal model for the metabolism of arsenic in human exist, this comparative study on different animal species suggests some conclusions on the mechanism of the intracellular retention of arsenic in mammalian tissues.

The interaction with cellular components is due to inorganic arsenic. Whereas the elimination and thus the detoxification of arsenic is closely related to the rate of its methylation in tissues (Vahter and Marafante, 1988).

From this brief analysis the “in vitro” toxicology research on As would be carried out considering the following aspects:

(i) the exposure, that must consider the inorganic and organic forms of As as identified in the human exposure.

(ii) the biochemical mechanisms of toxicity which are responsible for the toxic effects of As.

MATERIALS AND METHODS

3.1. Experiments on metal dermatotoxicity

3.1.1. Chemicals

Silver nitrate [$AgNO_3$], aluminium nitrate [$Al(NO_3)_3$], gold (III) chloride [$AuCl_3$], boric acid [H_3BO_3], beryllium chloride [$BeCl_2$], chromium (III) chloride [$CrCl_3$], germanium (IV) oxide [GeO_2], ammonium hexachloriridate [$(NH_4)_2IrCl_6$], molybdenum [$(H_4)Mo_7O_{24}$], lead (II) nitrate[$Pb(NO_3)_2$], platinum (II) chloride [$PtCl_2$], platinum (IV) chloride [$PtCl_4$], potassium antimoniate [$KSbO_7$], stannous chloride [$SnCl_2$], ammonium tellurate [$(NH_4)_2TeO_4$] and sodium tellurite [Na_2TeO_3], were purchased from ALFACHEM Cologno Monzese.

Barium nitrate [$Ba(NO_3)_2$], bismuth nitrate [$Bi(NO_3)_3$], cadmium sulphate [$3CdSO_4$], copper sulphate [$CuSO_4$], gallium nitrate [$Ga(NO_3)_3$], mercury chloride [$HgCl_2$], methyl mercury [CH_3HgCl], lithium nitrate [$LiNO_3$], manganese sulphate [$MnSO_4$], nickel sulphate [$NiSO_4$], ammonium chlorosmate [$(NH_4)_2OsCl_5$], rubidium chloride [RbCl] and ammonium chlororodite [$(NH_4)_3RhCl_6$], were purchased from ALFA Johnson Mattey, Karlsruhe.

Sodium selenite [Na_2SeO_3], titanocene [$(C_5H_5)_2TiCl_2$] and vanadocene [$(C_5H_5)_2VCl_2$] were purchased from SIGMA Chimica Divisione of SIGMA-ALDRICH, Milan.

Sodium chromate [Na_2CrO_4], Indium (III) chloridehydrate [$InCl_3$], ammonium terachloropalladate [$(NH_4)_2PdCl_4$], ammonium exachloropalladate [$(NH_4)_2PdCl_6$], ammonium hexacholoplatinate [$(NH_4)_2$ Pt Cl_6], ammonium tetracholoplatinate [$(NH_4)_2$ Pt Cl_4], and sodium selenate (VI) [Na_2SeO_4] were purchased from Fluka Divisione of SIGMA-ALDRICH, Milan.

Copper sulphate [$CuSO_4$] and lanthanum nitrate [$La(NO_3)_3$] were purchased from Merck, Darmstad, FGR.

3.1.2. HaCaT cell line

HaCaT cells (Human adult. Low Ca^{2+}, high Temperature) have been kindly provided by Professor Norbert E. Fusenig, DKFZ (Deutsches Krebsforschungzentrum), Im Neuenheimer Feld, Heidelberg (Deutschland).

Cultures of the established HaCaT cell line were routinely grown under standardised conditions in a Dulbecco's Modified Eagle's Medium (DMEM) with high glucose content, with sodium pyruvate, L- glutamine (CELBIO, Milan, Euroclone) supplemented with 10% Foetal Clone II serum (CELBIO, Milan, Hy Clone), 5 ml L- glutamine (Gibco, BRL, Milan), 5 ml of penicillin/streptomycin (10000 IU ml^{-1} and 10000 UG ml^{-1}, Gibco, BRL, Milan) in a humidified atmosphere (37°C and 5% CO_2).

Cultivation and expansion. Figure 3.1 shows how to culture HaCaT cell line.

The cells have been plated every third day adopting this procedure:

- The cells were taken out from the liquid nitrogen
- The vial of interest was warmed up in a 37°C bath
- When melted, the cells were transferred immediately into a 15ml Falcon tube and 10 ml of medium were added to the tube
- The cells were re- suspended
- The cell suspension was transferred into a T75 flask.

Concerning the cell expansion another procedure has been adopted:

- The cells were washed with a pre- warmed (37°C bath) PBS (10 ml) twice

- The PBS was removed and pre- warmed (37°C bath) trypsin (1 ml) was added to the T75 flask
- After few minutes 10 ml of medium were added to the flask to inactivate the trypsin
- The cell suspension was then collected into a 15 ml Falcon tube
- The cells were counted using a Neubauer chamber
- Calculation were made to obtain 500000 cells/T75 flask each Monday and 300000 cells/ T75 flask each Thursday (Monday and Thursday were chosen as days of plating)
- The cell suspension was transferred to the T75 flask containing 10 ml of medium

Freezing and thawing of cells. Cells were grown in flasks to 80% confluency. Thereafter, they were harvested, and the cell pellet was re- suspended in freezing medium (60% DMEM, 20% heat- inactivated serum, 20% DMSO) at 1 or 2 x 10^6 cells/ml. 1 ml aliquots were added to each freezing vial. Freezing vials wee put into a freezing container, which was transferred to -80°C. The next day, the vials were moved to the liquid nitrogen container. To de- freeze cells in the vials these were quickly de- frozen in a 37°C water bath, resuspended in 10 ml of the required volume of growth medium to dilute the DMSO, pelletted, resuspended in growth medium and plated out.

Figure 3.1- Cultivation and expansion protocol for HaCaT cell line to be exposed to metal compounds.

DAILY CULTIVATION				
t=0	1d	4d	5d	8d
Thawing of a vial stored in liquid N_2 ($1.5x10^6$/T75 flask)	Change of the medium	Trypsinisation and count	Change of the medium	Cells available for metal exposure
		$0.3x10^6$ cells/ T75 flask		$0.5x10^6$ cells/ T75 flask
		10ml DMEM+10% FBS		10ml DMEM+10% FBS

Colony Forming Efficiency Test (CFE). Standardised experimental conditions used in this cytotoxicity assay were described by Bertolero et al., (1987). Cells were plated in 60 mm tissue culture dishes (COSTAR, Cambridge, MA) in 4 ml of complete culture medium (200 cells per dish, six dishes per experimental point) and allowed to attach for 24h. The cells were then changed with medium containing selected concentrations of different metal salts and incubated for 24h and/ or 72h. After exposure, dishes were fed fresh medium and 7 days later the cultures were fixed with 10% formaldehyde in PBS (v/v) and stained with 10% Giemsa solution. Colonies containing > 50 cells were scored and the relative colony-forming efficiency (CFE) was expressed by the number of colonies in the treated cultures as a percent of those obtained in untreated control cultures (CFE= colonies/ cells plotted x 100) (Fig. 3.2).

Figure 3.2- Cytotoxicity assay protocol.

DAILY TREATMENT				
t=0	1d	4d		7d
200cells/60mm dish 5ml DMEM+ 10 % FBS	Metal exposure	Change of the medium	Two changes of medium	· Fix and stain · CFE

3.2. Experiments on metal neurotoxicity

3.2.1. Chemicals

Silver nitrate [$AgNO_3$], aluminium nitrate [$Al(NO_3)_3$], sodium hexafluoroarsenate (V) [$NaAsF_6$], potassium permanganate [$KmnO_4$], manganese (II) chloride [$MnCl_2$], sodium tellurite [Na_2TeO_3], sodium tellurate [Na_2TeO_4], and sodium vanadium oxide [$NaVO_3$] were purchased from ALFACHEM Cologno Monzese.

Cadmium sulphate [$3CdSO_4$] and methyl mercury [CH_3HgCl], were purchased from ALFA Johnson Mattey, Karlsruhe.

Sodium selenite [Na_2SeO_3], was purchased from SIGMA Chimica Divisione of SIGMA-ALDRICH, Milan.

Sodium meta arsenite (III) [$NaAsO_2$], sodium chromate [Na_2CrO_4], ammonium hexacholoplatinate [$(NH_4)_2$ Pt Cl_6], ammonium tetracholoplatinate [$(NH_4)_2$ Pt Cl_4] and ammonium hexachlororhodate [$(NH_4)_3RhCl_6$] were purchased from Fluka Divisione of SIGMA-ALDRICH, Milan.

Dimethylarsinic acid [$(CH_3)_2AsO(OH)$, DMA) was purchased from Merck, Darmstad, FGR.

3.2.2. PC12 cell line

The name of this cell line (PC12-p53, rat pheochromocytoma) means that the cells are engineered modified and contain in their DNA gene p53. Tet- on (tetracycline absent, expression of p53 gene) medium and Tet- off (tetracycline present, no expression of p53 gene) medium were used for the experimentations. This cell line was grown at 37°C in a humidified incubator with 5% CO_2. Due to the poor adherence of Pc12 cell lines, these cell lines were grown on collagen Vitrogen 100- coated tissue culture flasks and dishes during maintenance and experiments. The appropriate volume of collagen solution (1% (v/v) collagen Vitrogen 100; 1% (v/v) BSA 10%; 98% HBS (122mM NaCl; 2.67 mM KCl; 9.4mM glucose; 14 mM NaH_2PO_4;20 mM Hepes; pH 7.5)) was added to the dish or flask and the coating was performed for at least 3 hours at 37°C or overnight at room temperature under the laminar flow hood. The solution was then removed by aspiration and the coated flask was used immediately.

Cultivation and expansion. Figure 3.3 shows how to culture PC12-p53 cell line.

The cells have been plated every third day adopting this procedure:

- T75 flasks were coated by adding a coating solution made of HBSS (9.8 ml); Vitrogen 100 (100 µl) and BSA 10% (100µl). The flasks were then left in a humidified atmosphere (37°C, 5% CO_2) for 3 h
- Vials of the cells were taken out from the liquid nitrogen
- The vials were warmed up in a 37°C bath
- When melted, the cells were transferred immediately into a 15 ml Falcon tube and 10 ml of RPMI medium was added
- The tube with the cells was centrifuged (600 rpm for 10 minutes) at room temperature

- The supernatant was discarded
- The cells were resuspended in 10 ml pre- warmed (37°C) medium
- The collagen removed from T75 flasks and 5 ml of medium immediately added to the flasks
- The cell suspension was then transferred into T75 flasks in order to have a final volume of 15 ml/ T75 flask

During maintenance, cell lines were grown on T75 tissue culture flasks with 15 ml of medium, and subdivided twice per week with a subdivision ratio of 1:3. PC12-p53 cells were grown in RPMI Tet-Off selection medium (85% RPMI 1640 with GLUTAMAX I; 10% (v/v) heat- inactivated Horse Serum (HS); 5% (v/v) heat- inactivated Tet System approved foetal calf serum (FCS); 100 units/ ml penicillin streptomycin; 100μg/ml geneticin (G418), hygromycin B stock 50 mg/ml, tetracycline stock 1 mg/ml). To detach PC12- p53 cells the medium was aspirated, the cells were washed with Hanks' balanced salt solution (HBSS), then detached by the addition of the required amount of 1X trypsin/ EDTA. After 2 minutes of incubation the cells were dislodged. The trypsin was inactivated by the addition of the appropriate amount of RPMI Tet- Off selection medium. If appropriate, the cells were then counted, transferred to a 15 ml polypropylene tube and pelletted at 600 rpm (Beckman S4180 rotor) for 10 minutes at room temperature. The pellet was resuspended in the appropriate volume of RPMI medium.

Freezing and thawing of cells. Cells were grown in flasks to 80% confluency. Thereafter, they were harvested, and the cell pellet was re- suspended in freezing medium (90% FBS, 10% DMSO) at 1 or 2 x 10^6 cells/ml. 1 ml aliquots were added to each freezing vial. Freezing vials wee put into a freezing container, which was transferred to -80°C. The next

day, the vials were moved to the liquid nitrogen container. To de- freeze cells in the vials these were quickly de- frozen in a 37°C water bath, resuspended in 10 ml of the required volume of growth medium to dilute the DMSO, pelletted, resuspended in growth medium and plated out.

Figure 3.3- Cultivation and expansion protocol for PC12 cell line.

DAILY CULTIVATION		
t=0	4d	7d
Thawing of a vial stored in liquid N_2 ($1.5x10^6$ cells/T75 flask)	Trypsinisation and count	Cells available for metal exposure
	10^6 cells/T75 flask previously "coated" by collagen 15ml RPMI+ 10% HS+ 5% Tet System FCS	10^6 cells/T75 flask previously "coated" by collagen 15ml RPMI+ 10% HS+ 5% Tet System FCS

MTT test. Standardised experimental conditions used in MTT test were described by Mosmann, (1983). Cells were plated in 96 well plates (Costar, Cambridge, MA) previously treated with collagen (1% (v/v) collagen Vitrogen 100; 1% (v/v) BSA 10%; 98% HBS (122mM NaCl; 2.67 mM KCl; 9.4 mM glucose; 14 mM NaH_2PO_4; 20 mM Hepes:pH 7.5)) for 3 hours at 37°C to let the cells adhere to the plastic of the plate (10000 cells/96 well plate). After 24h, the medium was removed and the solution of the metal of interest was added to each well. The metal was left 24h and/or 72h depending on the exposure time chosen. Thereafter the MTT solution (MTT 1mg/ ml of medium) was directly added to each well and the 96 well plate incubated for 3 hours at 37°C and 5% CO_2. Thereafter the medium was discarded and a solution DMSO/ethanol 100% (v/v) was added to the cells to solubilise the formazan crystals. The plate was then put on a shaker and read at the

spectrophotometer at the wavelength of 570 nm with a reference wavelength of 630 nm (Fig.3.4).

Figure 3.4- MTT test protocol.

DAILY TREATMENT		
t=0	1d	2 (or 4) d
10^4 cells/ 96 wells plate 200µl RPMI+ 10% FCS+ 5% Tet system FCS	Metal exposure	· Add MTT solution · Incubation for 3h, 37°C, 5% CO_2 · Discard medium · Add DMSO/EtOh 100% · Read

3.3. Experiments on metal embryotoxicity

3.3.1. Chemicals

Methyl mercury [CH_3HgCl], was purchased from ALFA Johnson Mattey, Karlsruhe.

3.3.2. Embryonic Stem Cells D3 cell line

Embryonic stem cells, D3 (derived from eight 129/ Sv+/+4- day blastocysts of mice) have been kindly provided by Prof. Rolf Kemler (Max Plank Institute, Freiburg, Germany).

Cultures of the ES cell line D3 were routinely grown under standardised conditions in a Dulbecco's Modified Eagle's Medium (DMEM) with glucose, L- glutamine and $NaHCO_3$ (Gibco, Cat. No. 41965- 039 (Europe) or Gibco, Cat. No. 11965- 092 (USA)) supplemented with 16% Foetal Calf Serum (HyClone), 2U/ml L- glutamine (Gibco, BRL, Milan), 6 ml of penicillin/streptomycin (50 U/ml and 50 µg/ ml, Gibco, BRL, Milan), 1% Non essential Amino Acid (NAA) (Gibco, BRL, Milan) and 0.1 mM of β-

Mercaptoethanol (Sigma) in a humidified atmosphere (37°C and 5% CO_2). The mouse ES cell line D3 is cultured permanently in the presence of LIF (leukaemia inhibitory factor). In the absence of LIF Es cells start to differentiate spontaneously.

Cultivation and expansion. Figure 3.5 shows how to culture Es D3 cell line.

The cells have been plated every second day adopting this procedure:

- Vials of the cells were taken out from the liquid nitrogen
- The vials were warmed up in a 37°C bath
- When melted, the cells were transferred immediately into a 15 ml Falcon tube and 10 ml of DMEM medium was added
- The tube with the cells was centrifuged (1000 rpm for 5 minutes) at room temperature
- The supernatant was discarded
- The cells were resuspended in 10 ml pre- warmed (37°C) medium
- The cell suspension was then transferred into a 10 cm Petri culture dish and 0.1 % of the final volume of LIF was added

The cells have been expanded adopting this procedure:

- From a 25 mm Petri dish cells were washed with pre- warmed (37°C bath) PBS (1ml)
- PBS was removed and pre- warmed trypsin was added (300µl)
- 1ml of medium (DMEM) was added to inactivate the trypsin
- The cell suspension was collected into a 50 ml Falcon tube
- The cells were counted using a Neubauer chamber
- Calculation were made to have 25000 cells/ml (Vf= 2.5 ml)

- The cell suspension was then transferred into a 25 mm Petri dish with medium (Vf= 2.5 ml)
- 25 µl of LIF were then added to the seeded plate

Freezing and thawing of cells. Cells were grown in flasks to 80% confluency. Thereafter, they were harvested, and the cell pellet was re- suspended in freezing medium (80% FCS, 20% DMSO) at 1 or 2 x 10^6 cells/ml. 1 ml aliquots were added to each freezing vial. Freezing vials wee put into a freezing container, which was transferred to -80°C. The next day, the vials were moved to the liquid nitrogen container. To de- freeze cells in the vials these were quickly de- frozen in a 37°C water bath, resuspended in 10 ml of the required volume of growth medium to dilute the DMSO, pelletted, resuspended in growth medium and plated out.

Figure 3.5- Cultivation and expansion protocol for ES D3 cells.

DAILY CULTIVATION				
t=0	1d	2d	3d	4d
Thawing of a vial stored in liquid N_2 ($1.5x10^6$ cells/10cm Petri dish)	Change of medium	Trypsinisation and Count 62500cells/ 25 mm dish 2.5ml DMEM+ 16 % FCS+ LIF	Change of medium	Trypsinisation and Count 62500cells/ 25 mm dish 2.5ml DMEM+ 16 % FCS+ LIF

Beating measurements. The capability of pluripotent embryonic stem cells (ESC) to differentiate “in vitro” into different tissues provides an opportunity to develop an “in vitro” assay for screening chemicals for their embryotoxic potential. The measurement of

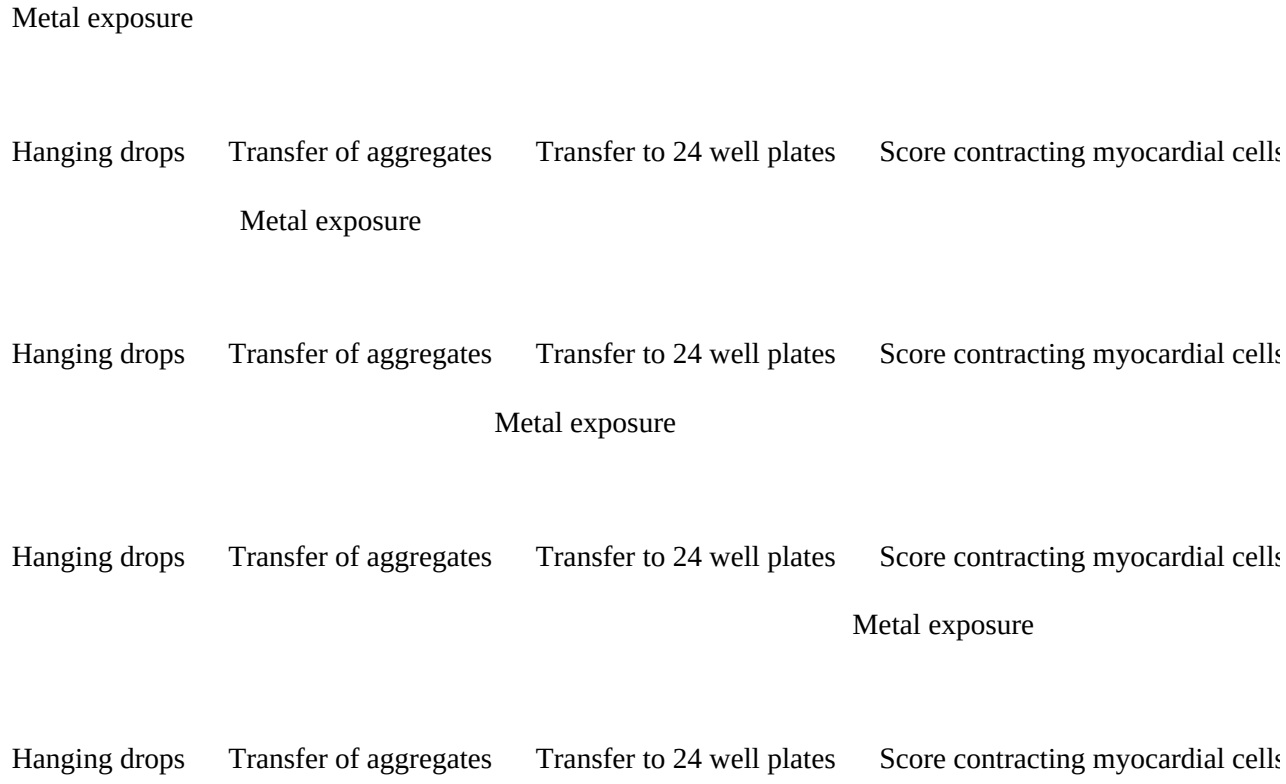
the “beat” was possible because of the differentiation of embryonic stem cells. The mouse ES cell line D3 is cultured permanently in the presence of LIF (leukaemia inhibitory factor). LIF is able to maintain ES cells in an undifferentiated stage. ES cells form embryoid bodies (Ebs) and differentiate under appropriate conditions into the major embryonic tissues when the leukaemia inhibitory factor is removed. Different concentrations of the test chemical are added to the embryonic stem cell suspension. Drops of ES cell suspension in supplemented DMEM are placed on the lids of 10 cm Petri dishes (“hanging drop” culture according to Wobus et al. 1991). After cultivation for 3 days the aggregates are transferred into bacterial (non tissue culture treated) petri dishes. 2 days later Ebs are placed into 24 well plates (tissue culture treated) where further development of Ebs proceeds into different embryonic tissues. Differentiation into contracting myocardial cells is determined by light microscopy after another 5 days of culture (Fig.3.6).

Fig 3.6- Beating test

DAILY TREATMENT			
t=0	3d	5d	10d
112500cells/10cm dish as HD 3ml DMEM+16% FCS	Transfer of aggregates	Transfer to 24 well plates	Score contracting myocardial cells

Preliminary experiments have been done treating the cells at different developmental stadium with methyl mercury, a known embryotoxic agent (Figure 3.7).

Figure 3.7-Different steps in which the cells have been treated with methyl mercury 10^{-6}M.



3.4 Experiments on metal carcinogenicity

3.4.1. Chemicals

Ammonium hexachloroplatinate [$(NH_4)_2PtCl_6$] , ammonium hexachloropalladate [$(NH_4)_2PdCl_6$], ammonium hexachlororhodate [$(NH_4)_3RhCl_6$] were purchased from Fluka Divisione of SIGMA-ALDRICH, Milan; ALFACHEM Cologno Monzese; SIGMA Chimica Divisione of SIGMA-ALDRICH, Milan; BRACCO, Milan; BDH, Milan.

Sodium arsenite ($NaAsO_2$) and arsenate (Na_2HAsO_4), monomethylarsonic acid ($CH_3As(OH)_2$, MMA) and dimethylarsinic acid ($(CH_3)_2AsO(OH)$, DMA) were analytical

grade commercial products which were supplied by Merck, Darmstad, FRG; Alfa products Danvers, MA; Sigma, St-Luis, Mo.

Trimethylarsine oxide (TMAO) was supplied by the Laboratory of Organic Chemistry, University of Liegi.

Arsenocholine [$(CH_3)_3AsCH_2CH_2OH$, trymethyl- (2- hydroxy- ethyl)- arsonium bromide, AsCh] and arsenobetaine [$(CH_3)_3AsCH_2COO^-$, carboxymethylemetrimethylarsonium bromide, Asβ] were synthesized according to the method of Goetz and Norin (1983).

3.4.2. BALB/3T3 cell line

BALB/3T3 cell line Cl A31-1- 1 (derived from subclones of the A31 clone, originallu isolated from embryonic mouse cells, family BALB/C) has been provided by the "Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia, Laboratorio Centro Substrati Cellulari di Brescia" (passage number 8/10).

Cultures of the established BALB/3T3 cell line were routinely grown in a Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Clone III serum (MEM- FBS, Flow Lab., Opera, Milan, Italy), with L-glutamine, penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) (Sigma Co, St. Louis, Mo). BALB/3T3 Clone A31- 1- 1 cell line has been grown in standardised conditions with a controlled atmosphere (5% CO_2, 37°C) in a HAEREUS incubator.

Cultivation and expansion. To maintain the cells in culture have been used: a) Dulbecco's PBS w/o calcium, magnesium and sodium bicarbonate (GIBCO BRL) to wash the cells; b) Trypsin EDTA solution (1X) (SIGMA cell culture, Milan) to trypsinise the cells; c) DMSO (Dimetyl sulphoxide) (SIGMA Hybri Max), sterile crio- tubes (NUNC) (INTER MED, San Giuliano Milanese (MI)), centrifuge ALC PK 120 (PBI, Milan) to freeze the cells.

To plate the cells have been used: sterile Petri dishes 100x 20 mm (FALCON, Cadorago (Co)); sterile tubes of 15 ml and 30 ml (FALCON); sterile pipettes of 1, 2, 5, 10 and 25 ml (FALCON); micropipettes P10, P20, P100, P200, and P1000, with relative tips (GILSON, Milan); an aspiration system made of a platinum- iridium tube (90% Pt and 10% Ir, 15 cm long, 2mm of diameter, 0,2 mm thick) (DEGUSSA, Pero (MI)) inserted into an aspirator (COSTAR, Concorezzo (MI)) and liked to a vacuum (MILLIPORE, Vinodrone (MI)).

To count the cells a Neubauer chamber, 0.0025 mm^2 (BLAU- BRAND) has been used. An optic microscope Olympus CK2 (OLYMPUS, Segrate (MI)) and crio- boxes (NALGENE, Milan) were needed.

The plating of the cells has been done in sterile conditions using sterile materials and working under laminar flow wood. The cells have been plated every third day adopting this procedure:

1. Remove the medium from the dish with semi confluent cells adhered
2. Wash cells with pre-warmed (37°C bath) PBS (10 ml) twice
3. Remove PBS and add pre- warmed (37°C bath) trypsin (1 ml)
4. Add 10 ml of medium to inactivate the trypsin
5. Collect the cell suspension into a 15 ml Falcon tube
6. Count the cells using a Neubauer chamber
7. Calculate 500000 cells/ Petri dish each Monday and 300000 cells/ Petri dish each Thursday

8. Transfer the cell suspension to the Petri dish containing 10 ml of medium

The number of cells present in each dish have been counted with a Neubauer chamber using this formula:

$$N= (a/b)x\ 10000\ x\ c$$

Where: N= n° of cells present in the solution;

a= n° of cells counted in b squares;

b= n° of squares counted (minimum three);

c= total volume of the solution expressed in ml.

The calculation of the ml of the solution needed to plate the desired number of cells has been obtained with this formula:

$$N: c= z: x$$

Where: N= total n° of cells present in the solution;

c= total volume of the solution expressed in ml;

z= total n° of cells to plate;

x= ml of the initial solution needed to plate the n° of cells needed.

Freezing and thawing of cells. Cells were grown in flasks to 80% confluency. Thereafter, they were harvested, and the cell pellet was re- suspended in freezing medium (80% DMEM, 10% heat- inactivated serum, 10% DMSO) at 1 or 2 x 10^6 cells/ml. 1 ml aliquots were added to each freezing vial. Freezing vials wee put into a freezing container, which was transferred to -80°C. The next day, the vials were moved to the liquid nitrogen container. To de- freeze cells in the vials these were quickly de- frozen in a 37°C water

bath, resuspended in 10 ml of the required volume of growth medium to dilute the DMSO, pelletted, resuspended in growth medium and plated out.

Transformation assay. BALB/3T3 clone A31- 1- 1 is intermediately susceptible to express transformation (Kakunaga et al., 1981). The neoplastic transformation assay and the criteria used for scoring transformed foci were those indicated by Kakunaga, (1973), Cortesi et al., (1983) and IARC/NCI/EPA Working group, (1985). Cells were plated in 60 mm tissue culture dishes in 4 ml of complete medium (10^4 cells per dish, 18 dishes/ experimental point). After a 24h attachment period the cells were treated for 72h with complete medium (DMEM) containing the adequate concentrations of metal salts as well as benzo (α) pyrene and NH_4VO_3 as positive controls. After the treatment, the cells were maintained in culture for 5 weeks, the culture media being renewed twice a week, then fixed with 10% formaldehyde in PBS (v/v) and stained with 10% Giemsa Blue solution. Control cultures were treated with the corresponding amount of bidistilled water only. Type III foci were scored in order to estimate the transformation frequencies which were defined as the number of type III foci per clonal survivor, as determined in the concurrent cytotoxicity assay (Fig 3.8).

Figure 3.8- Concurrent cytotoxicity and morphological transformation assay protocols concerning the determination of the carcinogenic potential of arsenic compounds in BALB/3T3 cell line.

CYTOTOXICITY				
t=0	1d	4d		7d
200 cells/ 60 mm dish 5 ml DMEM+ 10 % FBS	Metal exposure	Change of medium	Two changes of medium	· Fix and stain · CFE
MORPHOLOGICAL TRANSFORMATION				
t=0	1d	4d		5wk
10^4 cells/60 mm dish 5 ml MEM+ 10% FBS	Metal exposure	Change of medium	Change of medium (2 x wk)	· Fix and stain · CFE

The morphological transformation was quantitatively expressed by the transformation frequency (T_f) according to the equation:

$$T_f = \frac{\text{n° foci type III in all petri dishes}}{\text{n° dishes x } 10^4 \text{ x \% survival/ 100}}$$

3.4.3. Analytical determination of arsenic

Arsenic compounds. Sodium arsenite ($NaAsO_2$) and arsenate (Na_2HAsO_4), were analytical grade commercial products which were supplied by Merck, Darmstad, FRG; Alfa products Danvers, MA; Sigma, St-Luis, Mo.

Arsenocholine [[$(CH_3)_3AsCH_2$ CH_2OH, trymethyl- (2- hydroxy- ethyl- arsonium bromide, AsCh) and arsenobetaine [$(CH_3)_3AsCH_2COO^-$, carboxymethylemetrimethylarsonium bromide, Asβ] was synthesized according to the method of Goetz and Norin (1983).

Radiochemicals and radiolabelling. ^{74}As- arsenic carrier-free ($T_{1/2}$= 17 d) was prepared by proton activation of a metallic Ge target at the JRC- Ispra cyclotron. The radiochemical separation of the radiotracer from the irradiated target and the assessment of the radiochemical purity were reported elsewhere (Sabbioni et al., 1987).

^{74}As (III) and ^{74}As (V) solutions were prepared by adding the ^{74}As radiotracer in the appropriate trivalent or pentavalent form to aliquots of stable $NaAsO_2$ or Na_2HAsO_4 solutions. The oxidation states of the resulting inorganic solutions were tested by paper electrophoresis (>99.5 % in both cases).

Neutron activation analysis. Since Asβ and AsCh were not radiolabelled by ^{74}As, the study of the uptake of As in BALB/3T3 cells exposed to such compounds was carried out by neutron activation analysis (absolute limit of sensitivity 0.1 ng As) as previously described (Pietra et al. 1981). Briefly, intact cells (including unexposed cells as control) or their subfractions (pellet and cytosol) were placed in ultrapure quartz vials and freezed- dried. The sealed vials were then submitted to neutron irradiation in the HFR reactor (Petten, the Netherlands) in a thermal neutron flux of 2 x 10^{14} neutrons cm^{-2} sec^{-2}. Then, the induced ^{76}As was radiochemically separated from interfering radionuclides and its gamma- rays emitted (559.2 KeV) counted by computer- based high resolution gamma- ray spectrometry.

Counting of radioactivity. ^{74}As radioactivity was determined by integral γ counting (Philips PW 4800 Automatic Gamma Counter equipped with a 2 x 1 NaI (Tl) crystal). Each time radioactivity measurements of ^{74}As were interpreted in terms of exogenous metal concentration by comparing them with that of reference solutions of ^{74}As with known specific radioactivity.

Uptake of As in BALB/3T3 cells. Subconfluent cultures, growing in T 75 flasks, were exposed in 4 ml of complete culture medium to different concentrations of the metal compound tested. After different exposure times, the medium was removed and the adherent cells were washed three times with 20 ml PBS, and detached with a trypsine-EDTA solution (0.25 and 0.02 % w/ v respectively). In the uptake studies four flasks were used for each characterisation. Viable cells were counted and the metal uptake in intact cells was determined by two approaches: (a) measurement of the incorporation of ^{74}As (inorganic As (III) and As (V)) into the cells by integral-γ counting (see this section Radiochemicals and radiolabelling), (b) by neutron activation analysys (NAA) of the As associated to the cells after incubation with Asβ and AsCh (see the section Neutron Activation Analysis).

The intracellular repartition of the cellular metals between organelles (pellet fraction) and cytosol (supernatant fraction) was determined after disruption of cells by sonication and centrifugation at 105000 x g for 90 min in a refrigerated miniultracentrifuge TL 100 (Beckmann, Milan, Italy) to separate the organelle- free cytosol. Arsenic incorporated into pellets as well as in cytosols was determined by the two approaches above mentioned for the determination of total As in the intact cells. The experiments were repeated at least three times (Fig. 3.9).

Figure 3.9- Uptake of arsenic in BALB/3T3 cell line.

PROTOCOL		
t=0	1d	2 (or 4)d
Subconfluent cultures/T75 flasks 4 ml DMEM+10 % FBS	Metal exposure	· Wash with PBS · Detach with trypsin EDTA solution · Counting of ^{74}As (or NAA)

3.5. Statistical treatment

All the results obtained using the different cell lines, HaCaT, PC12, Es, and BALB/ 3T3 have been treated statistically using Student's t- test.

The parameters used for the statistical comparison were the number of type III foci (plate for each treatment) and the cell survival. The statistical analysis to determine whether the results for each treatment were significantly different from the experimental negative control was by Student's t- test with a significant level of $p<0.05$.

The relative equations are:

$$t = \frac{a - b}{\sigma_d}$$

a= average of the results obtained treating the cells with metal compounds

b= average of the controls

σ_d= average standard deviation:

$$\sigma_d = \sqrt{v_t/n_1 + v_t/n_2}$$

Where:

$$v_t = \_(x_1\text{-}a)^2+(x_2\text{-}b)^2/(n_1\text{-}1)+ (n_2\text{-}1)$$

The significance has been controlled by Student's t- values to evaluate the probabilities of 95% (p= 0.05) or 99% (p= 0.001).

If the experimental value is $t_{exp}>t_{theoretic}$, the difference of the results found compared to the controls is significant. On the contrary ($t_{exp}<$ t) the results of the two series can't be considered statistically different.

RESULTS

4.1 HaCaT cell line

The results obtained on HaCaT cell line concern: a) experiments on specific elements (platinum) and other 51 metal compounds at one dose exposure (100μM) for 72h b) setting of dose- effect relationship related to selected metal species as identified at the point a).

4.1.1. Study on different Platinum compounds

Tables 4.1 to 4.4 summarise the results concerning the cytotoxicity of different Pt compounds [$(NH_4)_2PtCl_4$, $(NH_4)_2PtCl_6$, $PtCl_2$ and $PtCl_4$] in HaCaT cells. The results are expressed as relative Colony Forming Efficiency (%).

The following conclusions can be drawn:

(i) A dose- dependent cytotoxicity response was observed after 72h exposure of HaCaT cells to concentrations of $PtCl_2$ and $(NH_4)_2PtCl_4$ ranging from 0.5 μM to 100 μM (Table 4.1).

(ii) Dose- dependent cytotoxicity responses were also observed in HaCaT cells after exposure to different concentrations of $PtCl_4$ and $(NH_4)_2PtCl_6$ ranging from 1 μM to 100 μM. (Table 4.2).

(iii) Time- dependent cytotoxicity responses were observed in the case of exposure of HaCaT cells to different Pt compounds such as $PtCl_4$, $(NH_4)_2PtCl_4$ and $(NH_4)_2PtCl_6$ to the concentration of 50 μM. (Table 4.3).

(iv) Cytotoxicity of HaCaT cells when treated with $PtCl_2$ at different concentrations seems to be more dose- dependent than time dependent. Exposure to 50 μM of the compound led to complete CFE inhibition already after 5 minutes. (Table 4.4).

Table 4.1 –Pt- induced cytotoxicity in HaCaT cells exposed to $PtCl_2$ and $(NH_4)_2PtCl_4$ for 72h.

Dose (μM)	CFE ± SD (% of the control)	
	$PtCl_2$	$(NH_4)_2PtCl_4$
Control	100	100
0.5	87.8± 3.1	
1	87.5± 9.1	97± 9.3
3	56.3± 2.1	-
5	18.7± 4.3	99.3± 8.3
7	7.1± 2.5	-
10	0	82.7±11.3
30	0	90.5± 13.2
50	0	75.8± 9.7
100	0	41.9± 8.3

Table 4.2 Pt- induced cytotoxicity in HaCaT cells exposed to $PtCl_4$ and $(NH_4)_2PtCl_6$ for 72h.

Dose (μM)	CFE ± SD (% of the control)	
	$PtCl_4$	$(NH_4)_2PtCl_6$
Control	100	100
1	93.2± 15.3	79.6± 3.4
5	98.1± 13.7	69.8± 4.3
10	89.4± 11.2	43.2± 6.5
30	51.6± 9.2	16.8± 2.5
50	2.4± 0.9	2.5± 0.8
100	0	0

Table 4.3 – Pt- induced cytotoxicity in HaCaT cells exposed to 50μM of $PtCl_4$, $(NH_4)_2PtCl_4$ and $(NH_4)_2PtCl_6$ for different times.

Exposure (h)	CFE ± SD (% of the control)		
	$PtCl_4$	$(NH_4)_2PtCl_4$	$(NH_4)_2PtCl_6$
Control	100	100	100
0.5	79.9± 2.1	86.2± 3.5	81.2± 9.3
1	-	90.5± 5.5	-
3	80.3± 11.7	81.3± 12.9	56± 6.7
24	62.5± 2	64± 1.7	10± 1.6
48	78.8± 4.4	72.3± 13.7	8.8± 2.2

Table 4.4- Pt- induced cytotoxicity in HaCaT cells exposed to $PtCl_2$ for different times.

Exposure	CFE ± SD (% of the control)		
(h)	0.1 μM	1 μM	50 μM
Control	100	100	100
5 minutes	-	-	0
10 minutes	74.1± 1.3	91.5± 4.6	0
15 minutes	-	90.4± 1.1	0
0.5	82.2± 8.6	83.6± 6.9	1.8± 1.2
1	-	-	0
6	-	-	0.6± 0.1
24	-	-	0
48	-	-	0

4.1.2. Systematic study of the cytotoxic effect of 51 metal compounds.

Tables 4.5,4.6 and 4.7 show the results of the screening related to the cytotoxic effect in HaCaT cells induced by 100μM exposure to 51 metal compounds.

The results obtained allowed to classify the metal compounds in three groups:

· Group 1: metal compounds with a cytotoxicity included between 106% [Tl_2SO_4] and 78.2% [$CoCl_2$ x $6H_2O$] (Table 4.5).

· Group 2: metal compounds with a cytotoxicity included between 68.5% [K_2TiO_3] and 36.2% [Na_2TeO_4] (Table 4.6).

· Group 3: metal compounds with a cytotoxicity included between 31.6% [$MnSO_4$ x $5H_2O$] and the total inhibition of growth ($AgNO_3$., $3CdSO_4$ x $8H_2O$, $CdMoO_4$, Na_2CrO_4, $CuSO_4$ x $5H_2O$, CH_3HgCl, $HgCl_2$, $(NH_4)_2PtCl_6$, $VOSO_4$ x $5H_2O$, NaVO3 x H2O and $(C_5H_5)_2VCl_2$) (Table 4.7).

Table 4.5- Cytotoxicity induced by exposure of HaCaT cells for 72h to 100μM of 26 metal compounds (Group 1).

Metal compound	CFE ± SD (% of the control)
Control	100
$Al(NO_3)_3 \times 9H_2O$	92.6± 6.6
H_3BO_3	96.2± 4.8
$Ba(NO_3)_2$	79.0± 5.7
$BeCl_2$	89.4± 1.4
$CoCl_2 \times 6H_2O$	78.2± 11
$CrCl_3$	88.9± 7.9
GeO_2	101± 7.1
$InCl_3$	96.1± 9.5
$(NH_4)_2IrCl_6$	93.9± 6.4
$La(NO_3)_9 \times 6H_2O$	104± 11
$LiNO_3 \times 3H_2O$	84.0± 16
$H_4Mo_7O_{24} \times 4H_2O$	99.0± 3.5
$NiSO_4 \times 7H_2O$	81.5± 2.1
$(NH_4)_2OsCl_6$	95.1± 3.2
$(NH_4)_2PdCl_4$	88.7± 2.4
$(NH_4)_2PdCl_6$	83.2± 1.4
RbCl	78.5± 0.7
$(NH_4)_3RhCl_6$	81.8± 16
$KSbO_7$	86.7± 10
Na_2SeO_4	88.5± 16
$SnCl_2 \times 2H_2O$	93.7± 7.0
$Th(NO_3)_4 \times H_2O$	100± 12
Tl_2SO_4	106± 10
$UO_2(NO_3)_2 \times 6H_2O$	97.7± 3.0
$Na_2WO_4 \times 2H_2O$	95.6± 11
$ZnSO_4 \times 7H_2O$	80.5± 12

Table 4.6- Cytotoxicity induced by exposure of HaCaT cells for 72h to 100μM of 7 metal compounds (Group 2).

Metal compound	CFE ± SD (% of the control)
Control	100
$Bi(NO_3)_3$	53.5± 7.8
$(NH_4)_2PtCl_4$	58.4± 8.5
Na_2TeO_4	36.2± 10
K_2TiO_3	68.5± 12
$TiCl_2$	55.7± 0.0
$(C_5H_5)_2TiCl_2$	54.3± 17
$Zr(NO_3)_4$	65.3± 16

Table 4.7- Cytotoxicity induced by exposure of HaCaT cells for 72h to 100μM of 18 metal compounds (Group 3).

Metal compound	CFE ± SD (% of the control)
Control	100
$AgNO_3$	0.0
$AuCl_3$	14.9± 11
$3CdSO_4$ x $8H_2O$	0.0
$CdMoO_4$	0.0
Na_2CrO_4	0.0
$CuSO_4$ x $5H_2O$	0.0
$Ga(NO_3)_3$ x $6H_2O$	10.9± 12
CH_3HgCl	0.0
$HgCl_2$	0.0
$KMnO_4$	4.20± 2.5
$MnSO_4$ x $5H_2O$	31.6± 6.1
$Pb(NO_3)_2$	10.7± 0.9
$(NH_4)_2PtCl_6$	0.0
Na_2SeO_3	17.8± 5.3
$(NH_4)_2TeO_4$	30.5± 4.9
Na_2TeO_3	3.00± 5.5
$VOSO_4$ x $5H_2O$	0.0
NaVO3 x H2O	0.0
$(C_5H_5)_2VCl_2$	0.0

4.1.3. Dose- effect study on selected metals.

For the metal species that exhibit almost 100 % of growth inhibition ($AgNO_3$, $3CdSO_4$ x $8H_2O$, $CuSO_4$ x $5H_2O$, CH_3HgCl, $HgCl_2$ and $MnSO_4$), experiments have been carried out using different concentration to set dose- effect relationships and to establish the IC_{50} (Tables 4.8 to 4.10).

The intervals of concentrations in which IC_{50} take place are:

a) CH_3HgCl: 1µM to 3µM (Table 4.10).

b) $AgNO_3$: 7µM to 10µM (Table 4.8).

c) $3CdSO_4$ x $8H_2O$, $CuSO_4$ x $5H_2O$, $HgCl_2$ and $MnSO_4$ x $5H_2O$ 10µM to 100µM (Tables 4.8, 4.9 and 4.10).

Table 4.8- Cytotoxicity induced by different concentrations of $AgNO_3$ and $3CdSO_4$ x $8H_2O$ in HaCaT cells exposed for 72h.

Dose (µM)	CFE ± SD (% of the control)	
	$AgNO_3$	$3CdSO_4$ x $8H_2O$
Control	100	100
0.1	72.0± 10	76.1± 5.6
0.5	94.3± 3.4	96.4± 7.5
1	80.7± 7.9	82.3± 5.5
3	87.4± 0.7	76.9± 3.8
5	83.6± 3.9	79.5± 1.5
7	83.5± 4.0	81.0± 5.5
10	30.7± 5.2	61.1± 0.7
100	0	0

Table 4.9- Cytotoxicity induced by different concentrations of $CuSO_4$ x $5H_2O$ and $MnSO_4$ x $5H_2O$ in HaCaT cells exposed for 72h.

Dose (μM)	CFE ± SD (% of the control)	
	$CuSO_4$ x $5H_2O$	$MnSO_4$ x $5H_2O$
Control	100	100
0.1	68.6± 7.5	103± 2.1
0.5	84.4± 2.0	100± 12
1	93.3± 1.9	92.2± 4.7
3	94.8± 8.2	94.1± 0.4
5	89.3± 0.7	89.2± 0.9
7	82.6± 13	89.6± 3.4
10	81.3± 1.5	91.5± 6.4
100	0	31.6± 6.1

Table 4.10- Cytotoxicity induced by different concentrations of $HgCl_2$ and CH_3HgCl in HaCaT cells exposed for 72h.

Dose (μM)	CFE ± SD (% of the control)	
	$HgCl_2$	CH_3HgCl
Control	100	100
0.1	81.4± 14	90.6± 0.8
0.5	89.8± 0.8	86.7± 5.8
1	79.1± 11	60.1± 3.5
3	68.5± 10	37.7± 1.6
5	72.6± 4.7	0
7	69.2± 4.4	0
10	60.6± 7.6	0
100	0	0

4.2. PC12 cell line

The results obtained on PC12 cell line concern experiments on the induction of cytotoxicity by exposure of cells for 24 and 72h to 24 metal compounds into two different growth media (Tet- off and Tet- on).

4.2.1. Systematic study of the cytotoxic effect of metal compounds

Table 4.11 summarise the results of the screening related to the cytotoxic effect in PC12 cells induced at 24h post exposure by 14 metal compounds at the concentration of 100μM. The screening has been carried out with two media (Tet- on and Tet- off), in relation to the expression of p53 (see chapter 3 Materials and Methods).

The results are expressed as % viability as determined by MTT test.

The following conclusion can be drawn:

(i) The MTT incorporation into cells treated with DMA, Au, B, Mn, Pd, Pt, Rh, Te and Tl shows similar values using the two different growth media. Among the element tested Te (oxidation state IV) showed the highest cytotoxicity (10.4 % of the control in Tet- on medium and 11.6 % in Tet-off medium).

(ii) The MTT incorporation in cells treated with Al, CH_3HgCl and Sn was higher if cells were treated with Tet-on medium being 105, 27.3 and 100 % and 67.5, 3.6 and 8.7 % of the control in Tet- off medium respectively.

(iii) The MTT incorporation in cells treated with As and Hg in Tet- on medium was lower compared to the incorporation in Tet- off medium being 38.7 and 21.7 % of the control (Tet- on) and 75.9 and 82 (Tet- off) respectively.

Being Tet- on medium apparently more sensitive in relation to the cytotoxic response another screening of different metal compounds using the fixed concentration of 100μM at

exposure periods of 24 and 72h was carried out. Table 4.12 shows the results of such screening on 24 metal compounds.

The following conclusion can drawn:

(i) The MTT incorporation in cells treated with Ag, As (III) as $NaAsO_2$ (but not $NaAsF_6$), Cd, Cr, Hg and Pt (IV) was higher at 24h in comparison with the incorporation found at 72h being 36.0, 38.7, 37.0, 15.8, 21.7 and 73.5 and 4.20, 12.2, 10.9, 7.00, 5.10 and 20.5 respectively.

(ii) The MTT incorporation in cells treated with the other metals shows similar values at different exposure times.

Table 4.11- Cytotoxicity screening of 14 metal compounds in PC12 cells exposed for 24h at the concentration of 100μM with Tet- off and Tet- on selection media.

Metal compounds	Survival fraction (% of the control) ± S.D.	
	Tet- on medium	Tet- off medium
Control	100	100
$Al(NO_3)_3$	105± 1.0	67.5± 1.2
$NaAsO_2$	38.7± 12	75.9± 1.3
DMA	100± 0.5	97.0± 1.2
$AuCl_3$	94.0± 1.3	90.0± 1.1
H_3BO_3	108± 1.0	101± 1.2
$HgCl_2$	21.7± 13	82.0± 1.3
CH_3HgCl	27.3± 15	3.60± 1.4
$MnCl_2$	81.4± 14	98.0± 1.1
$(NH_4)_2PdCl_6$	100± 1.1	95.0± 1.2
$(NH_4)_2PtCl_6$	73.4± 15	94.0± 1.1
$(NH_4)_2RhCl_6$	96.0± 1.0	95.0± 1.2
$SnCl_2$	100± 1.2	8.70± 1.4
K_2TeO_3	10.4± 1.2	11.6± 1.4
Tl_2SO_4	110± 1.1	107± 0.5

Table 4.12- Cytotoxicity induced by 24 metal compounds in PC12 cells exposed for 24 and 72h at the concentration of 100μM (Tet- on medium).

Metal compound	Survival fraction (% of the control) ± S.D.	
	24h	72h
Control	100	100
$AgNO_3$	36.0± 15	4.20± 4.0
$Al(NO_3)_3$	105± 1.0	100± 1.0
$NaAsO_2$	38.7± 12	12.2± 16
$NaAsF_6$	94.3±14	101±11
DMA	100± 0.5	98.5± 8.2
$AuCl_3$	94.0± 13	-
H_3BO_3	108± 1.0	-
$3CdSO_4$ x $8H_2O$	37.0±9.0	10.9± 10
Na_2CrO_4	15.8± 9.4	7.00± 7.0
$HgCl_2$	21.7± 13	18.1± 11
CH_3HgCl	27.3± 15	22.8± 2.5
$MnCl_2$	81.4± 14	91.0± 13
$KMnO_4$	33.3± 6.2	20.4± 9.5
$(NH_4)_2PdCl_6$	100± 1.0	-
$(NH_4)_2PtCl_4$	73.4± 15	61.2± 14
$(NH_4)_2PtCl_6$	73.5± 1.1	20.5± 16
$(NH_4)_2RhCl_6$	96.0± 1.0	-
Na_2SeO_3	73.8± 15	58.0± 12
$SnCl_2$	100± 1.2	-
Na_2TeO_3	14.3± 1.1	15.1± 2.4
K_2TeO_3	10.4± 1.2	-
Na_2TeO_4	43.1± 1.7	26.6± 13
Tl_2SO_4	110± 1.1	-
$NaVO_3$	35.6± 7.8	35.3± 11

4.2.2. Dose effect study on selected metals

Tables 4.13 to 4.20 summarise the results concerning the cytotoxicity of different metal compounds, different exposure times (24 and 72h) and different media (Tet On and Tet Off) in PC12 cells. The results are expressed as % viability as determined by MTT test.

The following conclusions can be drawn:

(i) There is no significant cytotoxic effect of $Al(NO_3)_3$ in On and Off medium at all different concentration tested (Tab. 4.13).

(ii) The cytotoxicity of $HgCl_2$ seems dose- dependent rather than time- dependent The type of medium influences that result only at concentrations up to 5μM (Tab. 4.14).

(iii) The cytotoxicity effect of CH_3HgCl is dose- dependent when cells were exposed for 24h in the presence of Tet On and Tet Off medium (Tab. 4.15).

At 72h the cytotoxicity is not dose dependent but equally distributed in the range of the concentrations tested (0.1 to 100μM).

(iv) When cells were exposed to Mn compounds ($MnCl_2$ and $KMnO_4$) no cytotoxicity was observed with the exception of 100μM exposure to $KMnO_4$ (Table 4.16).The values are lower when cells were treated in the presence of Tet Off medium (Tab. 4.17).

(v) No effect was shown when cells were treated with $SnCl_2$. The metal in the presence of Tet On medium shows a higher viability in comparison with Tet Off medium at both exposure times considered (Tab. 4.18).

(vi) A dose- dependent cytotoxicity response was observed after exposure of PC12 cells to concentrations of Na_2TeO_3 and Na_2TeO_4 ranging from 0.1μM to 100μM (Tabs. 4.19 and 4.20).

Table 4.13- Cytotoxicity induced by $Al(NO_3)_3$ in PC12 cells exposed to different concentrations of the metal for 72h with Tet- on and Tet- off media.

Dose	Survival fraction (% of the control) ± S.D.	
(μM)	Tet- on medium	Tet- off medium
Control	100	100
1	96.9± 10	97.6± 10
10	87.9± 9.0	85.8± 11
100	100.1± 6.0	82.2± 5.0
500	110.2± 4.0	97.4± 7.0

Table 4.14- Cytotoxicity induced by $HgCl_2$ in PC12 cells exposed to different concentrations of the metal for 24 and 72h with Tet- on and Tet- off media.

Dose	Survival fraction (% of the control) ± S.D.			
(μM)	Tet- on medium		Tet- off medium	
	24h	72h	24h	72h
Control	100	100	100	100
0.1	128± 7.9	128± 10	103± 0.0	-
1	101± 12	119± 11	100± 1.4	106± 15
3	125± 14	-	99.0± 11	85.3± 3.8
5	119± 13	118± 12	92.7± 16	94.2± 14
10	64.7± 10	59.3± 0.0	81.0± 11	-
30	23.6± 9.0	21.0± 9.0	48.5± 1.3	35.7± 2.5
50	18.9± 14	21.8± 8.0	-	24.3± 4.9
100	21.7± 13	18.1± 11	22.9± 11	19.2± 5.7

Table 4.15- Cytotoxicity induced by CH_3HgCl in PC12 cells exposed to different concentrations of the metal for 24 and 72h with Tet- on and Tet- off media.

Dose (μM)	Survival fraction (% of the control) ± S.D.			
	Tet- on medium		Tet- off medium	
	24h	72h	24h	72h
Control	100	100	100	100
0.1	132± 9.0	-	72.1± 16	21.4± 1.9
1	76.0± 14	19.5± 2.1	42.7± 0.1	21.6± 4.8
3	42.4± 10	16.1± 1.8	56.4± 10	16.3± 1.8
5	33.4± 3.4	15.9± 1.8	-	30.2± 4.7
10	20.4± 4.2	26.9± 3	59.1± 12	21.1± 5.7
30	26.2± 1.1	30.5± 3.5	37.0± 14	19.4± 1.9
50	22.0± 4.2	34.9± 4.1	37.3± 6.4	19.0± 5.4
100	27.3± 15	22.8± 2.5	38.7± 12	19.3± 7.5

Table 4.16- Cytotoxicity induced by $MnCl_2$ in PC12 cells exposed to different concentrations of the metal for 72h with Tet- on and Tet- off media.

Dose (μM)	Survival fraction (% of the control) ± S.D.	
	Tet- on medium	Tet- off medium
Control	100	100
0.1	106± 4.0	106± 2.0
0.3	137± 3.0	90.7± 3.0
0.5	127± 1.0	99.5± 10
1	131± 5.0	76.3± 11
3	104± 4.0	100± 9.0
5	135± 6.0	87.1± 6.0
10	87.7± 5.0	99.7± 5.0
100	91.0± 13	-

Table 4.17- Cytotoxicity induced by $KMnO_4$ in PC12 cells exposed to different concentrations of the metal for 72h with Tet- on and Tet- off media.

Dose	Survival fraction (% of the control) ± S.D.	
(μM)	Tet- on medium	Tet- off medium
Control	100	100
0.1	101± 1.0	99.0± 7.0
0.3	123± 4.0	97.3± 10
0.5	134± 3.0	75.5± 11
1	138± 2.0	69.9± 9.0
3	120± 4.0	66.1± 4.0
5	137± 5.0	71.0± 7.0
10	106± 8.0	95.1± 3.0
100	20.4± 9.5	-

Table 4.18- Cytotoxicity induced by $SnCl_2$ in PC12 cells exposed to different concentrations of the metal for 24 and 72h with Tet- on and Tet- off media.

Dose	Survival fraction (% of the control) ± S.D. Survival fraction (% of the control) ± S.D.			
(μM)	Tet- on medium		Tet- off medium	
	24h	72h	24h	72h
Control	100	100	100	100
0.1	120± 9.7	133± 12	81.2± 12	78.7± 16
1	104± 0.1	125± 13	73.0± 11	71.3± 12
3	107± 2.3	140± 12	83.1± 12	75.0± 11
5	109± 10	125± 12	72.9± 0.8	76.8± 13
10	110± 12	132± 15	85.1± 14	71.2± 14
30	103± 7.1	137± 12	87.8± 10	83.4± 11
50	94.8± 7.4	130± 13	95.1± 14	85.8± 14
100	100± 1.2	112± 12	100± 12	92.6± 7.1

Table 4.19- Cytotoxicity induced by Na_2TeO_3 in PC12 cells exposed to different concentrations of the metal for 24 and 72h with Tet- on and Tet- off media.

Dose (μM)	Survival fraction (% of the control) ± S.D.			
	Tet- on medium		Tet- off medium	
	24h	72h	24h	72h
Control	100	100	100	100
0.1	126± 12	100± 0.0	91.0± 8.1	61.4± 8.3
1	51.7± 0.7	64.2± 13	98.8± 12	49.8± 1.5
3	50.7± 0.1	59.4± 13	89.4± 10	54.8± 14
5	53.4± 2.2	54.6± 11	79.5± 14	37± 3.6
10	47.8± 15	43.6± 15	91.3± 14	31.0± 12
30	40.3± 13	34.6± 11	62.2± 13	31.6± 12
50	21.7± 1.1	20.6± 6.6	68.5± 16	18.7± 0.1
100	14.3± 1.1	15.1± 2.4	52.8± 4.5	33.4± 11

Table 4.20- Cytotoxicity induced by Na_2TeO_4 in PC12 cells exposed to different concentrations of the metal for 24 and 72h with Tet- on and Tet- off media.

Dose (μM)	Survival fraction (% of the control) ± S.D.			
	Tet- on medium		Tet- off medium	
	24h	72h	24h	72h
Control	100	100	100	100
0.1	108± 7.8	109± 12	109± 3.3	65.2± 14
1	125± 11	108± 9.5	114± 4.7	58.0± 11
3	92.4± 14	68.5± 13	65.8± 11	39.3± 5.5
5	127± 9.0	70.7± 8.4	63.3± 10	37.8± 6.6
10	111± 12	41.6± 8.5	38.5± 14	41.9± 3.2
30	42.5± 3.1	31.6± 11	44.6± 16	28.6± 10
50	41.5± 2.4	35.8± 11	49.4± 12	39.7± 14
100	43.1± 1.7	26.6± 13	48.5± 12	37.1± 5.3

Table 4.21 summarises the results concerning the cytotoxicity induced in PC12 cells by $NaAsO_2$ $NaAsO_2$, $NaAsF_6$, $(NH_4)2PtCl_4$ and $(NH_4)_2PtCl_6$ at concentrations ranging from 0.1μM to 500μM and at the exposure time of 72h, in the presence of Tet On medium.

The following conclusion can be drawn:

(i) A dose dependent cytotoxicity response was observed for $NaAsO_2$, $(NH_4)_2PtCl_4$ and $(NH_4)_2PtCl_6$.

(ii) No effect was observed for $NaAsF_6$.

Table 4.21- Cytotoxicity induced by $NaAsO_2$, $NaAsF_6$, $(NH_4)_2PtCl_4$ and $(NH_4)_2PtCl_6$ in PC12 cells exposed to different concentrations of metal compounds for 72h (Tet- on medium).

Dose (μM)	Survival fraction (% of the control) ± S.D.			
	$NaAsO_2$	$NaAsF_6$	$(NH_4)_2PtCl_4$	$(NH_4)_2PtCl_6$
Control	100	100	100	100
0.1	121± 4.1	133± 12	120± 9.7	118± 3.2
0.3	121± 3.9	125± 12	117± 0.1	103± 6.3
0.5	107± 3.5	135± 13	122± 2.4	117± 9.4
1	98.8± 14	92.0± 12	111± 9.6	106± 11
3	81.1± 6.9	102± 13	120± 3.1	112± 6.6
5	63.7± 7.7	112± 6.8	115± 16	112± 1.7
10	45.3± 8.3	95.1± 11	125± 15	97.5± 9.3
100	12.2± 16	101± 11	61.2± 14	20.5± 16
500	0	-	3.1± 1.0	0

4.3. Embryonic stem cells, D3

Results in this area concern preliminary experiments on methyl mercury chloride.

4.3.1. Study of the effect of methyl mercury by the ES Cell Differentiation Assay

Table 4.22 summarise the results of the effect of methyl mercury on the beating of Es D3 cells.

The following conclusions can be drawn:

(i) The "beating" in myocardial cells obtained when Es cells were treated with CH_3HgCl at the first step of the procedure (hanging drops) is only 30 % compared to the untreated cells (control).

(ii) Apparently no toxicity was found when cells were treated during the other steps of the procedure (aggregates, 24 well paltes, myocardial cells).

Table 4.22- Embryotoxicity induced by 1μM of CH_3HgCl as measured by "cardiac beat" in Es D3 cells.[a]

Dose (μM)	Measurement of the "cardiac beat" (% of the beats of the control)			
	Step of metal addition			
	Hanging drops	Aggregates	24 well plates	Myocardial cells
Control	100	100	100	100
1	30	119	100	100

a: for details see chapter Materials and Methods.

4.4. BALB/3T3 cell line

4.4.1. Uptake and intracellular repartition of As compounds in BALB/3T3 cells.

Figures 4.1 and 4.2 summarise the results of the uptake of As by BALB/3T3 cells after incubation with $NaAsO_2$, $NaHAsO_4$, Asβ and AsCh determined by ^{74}As labelling ($NaAsO_2$ and $NaHAsO_4$) or by NAA (Asβ and AsCh).

The following conclusions can be drawn:

(i) the rate of ^{74}As uptake by BALB/3T3 cells after incubation with either sodium arsenite or sodium arsenate was dose- dependent (Fig.4.1). Incubation for 3h with equimolar concentrations (10 μM) of As (III) or As (V) resulted in about 4- fold higher incorporation of As in cells exposed to As (III) as compared to As (V).

(ii) the uptake of As by cells after incubation for 24h with the different organo-arsenic species was in all cases linear with the increasing of dose exposure h (Figures 4.2). In particular the incorporation of As by the cells after incubation for 24h with Asβ or AsCh was also linear with the dose. Exposure to AsCh resulted in a higher uptake of As than for exposure to Asβ. In the experimental interval of concentrations tested the cellular uptake of As (on average) was 2.8-fold higher for AsCh than Asβ.

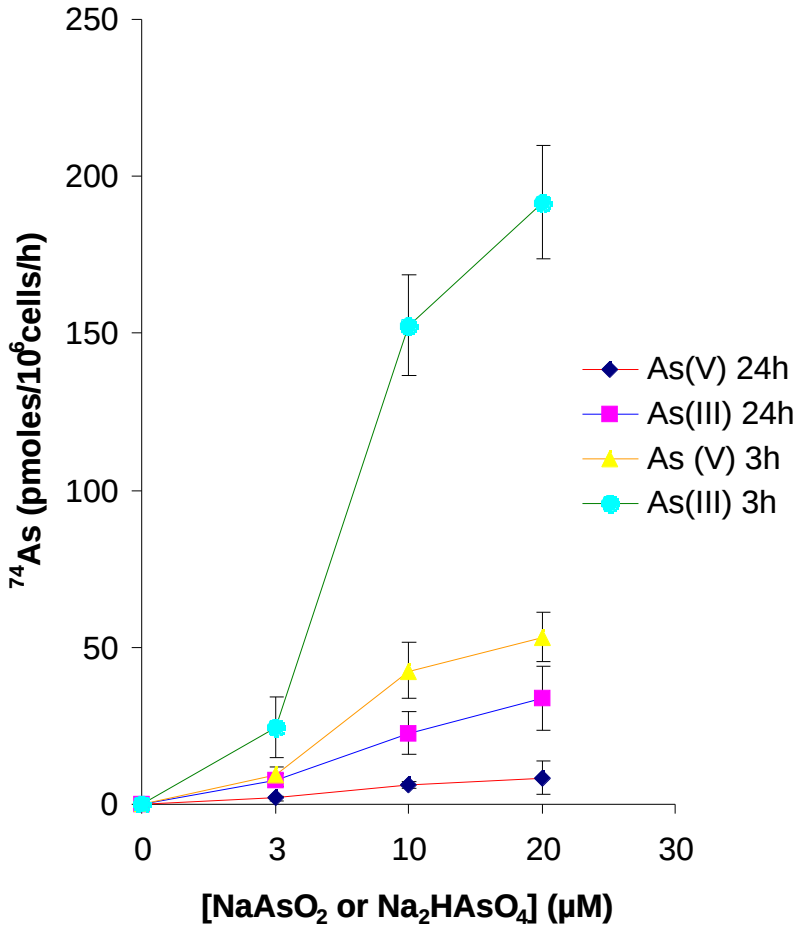


Fig. 4.1 Arsenic uptake by BALB/3T3 cells after exposure to ^{74}As-labelled [$NaAsO_2$] or [Na_2HAsO_4] as determined by incorporation of ^{74}As radiotracer. Cells were exposed for 3 and 24 h.

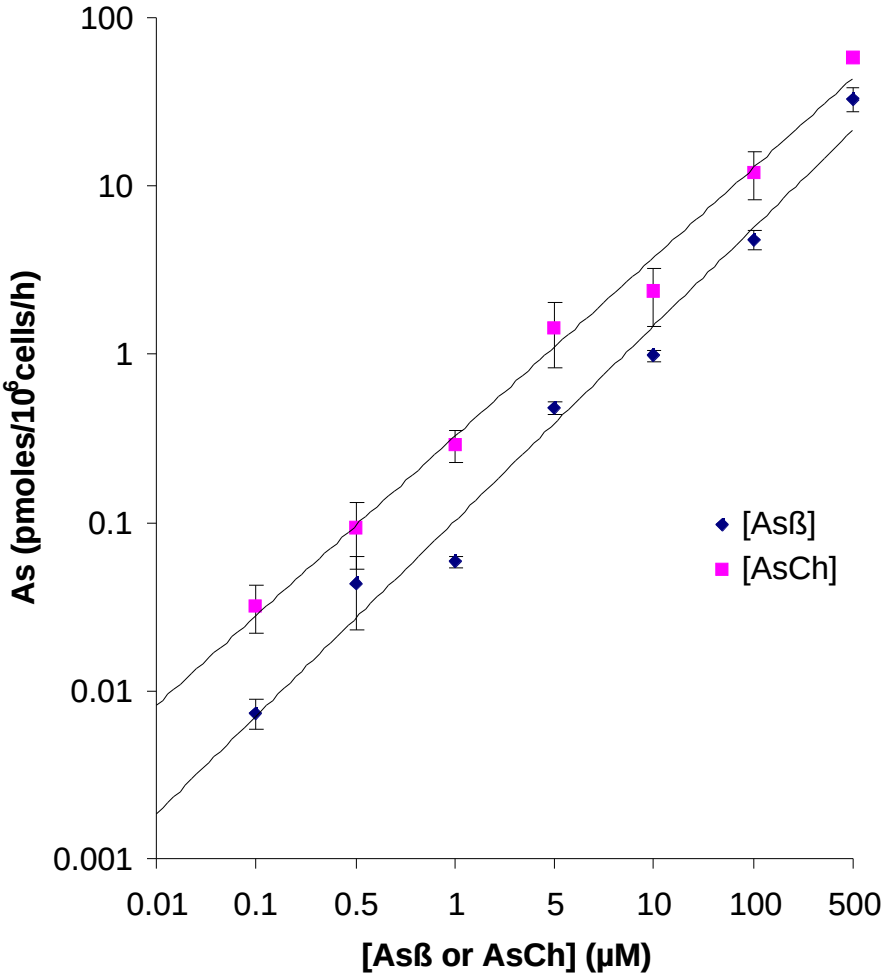


Fig. 4.2 Arsenic uptake by BALB/3T3 cells exposed to Asß or AsCh for 24 h as determined by NAA.

4.4.2. Neoplastic morphological transformation of BALB/3T3 cells by As compounds

Table 4.23 summarises the results of the concurrent cytotoxicity and morphological transformation assays carried out on BALB/3T3 cells exposed to different inorganic- ($NaAsO_2$ and $NaHAsO_4$) and organo- arsenic (Asβ and AsCh) compounds.

The following conclusions can be drawn:

(i) both inorganic trivalent and pentavalent As were found positive in the transformation assay, As (III) being more active than As (V) in inducing morphological transformation (transformation frequency of 4.9×10^{-4} at 5 μM of As (III) and 2.1×10^{-4} at 20 μM for As (V)).

(ii) Asβ and AsCh at concentrations of 500 μM failed to induce the formation of any type III foci.

(iii) the cytotoxicity data explored from the concurrent colony forming assay revealed once again the strong cytotoxic effect of $NaAsO_2$ and less extent of $NaHAsO_4$ compared to the not significant inhibition of CFE obtained with all other As-compounds.

Table 4.23- Concurrent cytotoxicity and morphological transformation induced by different As compounds in BALB/3T3 cells.

Exposure Compounds	Dose (μM)	CFE (%)	N° of type III foci/ N° of dishes	N° of type III positive dishes/ N° of dishes	$T_f \times 10^{-4}$	p
H_2O bid. (v/v)	-	100	0/18	0/18	0.0	-
$NaAsO_2$	5	7± 2.4	5/18	4/18	4.9	<0.05
$NaHAsO_4$	20	29± 3.8	9/18	7/18	2.1	<0.05
AsCh	500	95± 3.0	0/18	0/18	0.0	-
Asβ	500	94± 5.0	0/18	0/18	0.0	-

DISCUSSION

The findings of the present research show the great potential of the “in vitro” toxicity testing methods in investigations concerning the cytotoxic effects of trace metal compounds in different toxicological areas such as dermatotoxicity, neurotoxicity, embryotoxicity and carcinogenic potential as strategy point of “in vitro” metal toxicology studies. In particular, we adopted a strategy based on systematic screening study by exposing cells to a fixed dose of individual metal compounds. This strategy, is interesting because it allows to set a ranking of metal cytotoxicity that is a basic indication to give priorities to metal species in subsequent investigations related to the understanding of the molecular mechanisms of toxic actions of trace metals (Table 5.1)

Table 5.1.- Strategy to give priorities to metal compounds to be assayed by “in vitro” toxicity testing.

Step	Objective
1. Screening test at a fixed dose exposure of individual metal (general basal cytotoxicity)	- Establish the ranking of toxicity for a great number of metals. - Identify metal compounds with high toxicity and suggest priorities for subsequent dose- effect relationship studies.
2. Setting of dose- effect relationships on metal compounds as identified of first priority at the point 1.	Determine the IC_{50} and the experimental dose ranges of metal compounds to be used for subsequent mechanistic studies.
3. Establishing relations between metal exposures as investigated at the point 2 and specific toxicological endpoints.	Give the molecular basis to the toxicological effects induced by metal compounds in cellular systems.

HaCaT cell line

Skin is one of the first barrier against environmental and occupational exposure to metals and their compounds. Contact of some metal compounds with the skin can induce immediate or cell- mediated hypersensitivity dermatitis in general population and preferentially exposed subjects. In the present work, human immortalised HaCaT cell line, used as “in vitro” cellular model, provides excellent opportunity for studying metal-induced dermatotoxic effects. Studies on specific metal compounds such as Pt compounds were carried out to investigate the effect of two key parameters (speciation and time of exposure) on the cytotoxic response.

The data on the cytotoxicity (expressed as CFE) induced in HaCaT cells by different platinum compounds [$(NH_4)_2PtCl_4$, $(NH_4)_2PtCl_6$, $PtCl_2$ and $PtCl_4$] indicate that $PtCl_2$ and $(NH_4)_2PtCl_6$ are more cytotoxic compared to the other two platinum compounds [$PtCl_4$ and $(NH_4)_2PtCl_4$]. In particular Pt- exposed cells show the following ranking of cellular toxicity confirming the great influence of the chemical form of the metal on its cytotoxicity:

$$PtCl_2 > (NH_4)_2PtCl_6 > PtCl_4 > (NH_4)_2PtCl_4$$

Experiments concerning the cytotoxicity of these four platinum compounds show that time strongly influence the cytotoxic response of the metal (Tables 4.3 and 4.4). This finding that the cytotoxic action of Pt species on human keratinocytes is in agreement with some observations of the literature which proved that certain platinum complexes are cytotoxic to human fibroblasts “in vitro” (Cocco,1999). Furthermore, an antimitotic effect on mouse skin ”in vivo” indicates the potential therapeutic use of these compounds in the treatment of hyperproliferative skin diseases, such as psoriasis (Lugovic and Lipozencic, 1997). The data obtained in the present work are also in agreement with the potent sensitising and allergenic cations of platinum to humans. In particular, reaction of chloroplatinates,

particularly at the working environment, can be severe and include asthma, urticaria and allergic contact sensitisation (Kligman, 1966). Interestingly, the sensitisation to hexachloroplatinate in man is highly specific and distinct from tetrachloroplatinate, perfectly in agreement with our results (Mazzotti, 1999).

The systematic study (screening test) related to the cytotoxic effect induced by 51 metal compounds to fixed dose exposure of 100μM for 72h allowed a classification of the metal species tested as 3 Groups according to the degree of their cytotoxic response (Table 5.2). Then, on the setting of dose- effect relationships was studied on metal compounds with the highest degree of toxicity (Group 3, Table 4.7).

Table 5.2- Classification of metal compounds according to their cytotoxicity induced in the HaCaT cell line.

Group	Range of inhibition of CFE (% of the control)	Number of metals
I (No or little inhibition)	100- 80	26
II (Moderate inhibition)	70- 35	7
III (Strong inhibition)	35- 0	18

A dose dependent cytotoxicity response was observed for the following salts: $AgNO_3$, $3CdSO_4$ x $8H_2O$, $CuSO_4$ x $5H_2O$, CH_3HgCl, $HgCl_2$ and $MnSO_4$ x $5H_2O$. In any case the IC_{50} was between 10μM and 100μM (Tables 4.8, 4.9 and 4.10) with the exception of CH_3HgCl where the inhibition concentration was estimated between 3μM and 5μM (Table 4.10).

The screening of cytotoxicity of different metal compounds in HaCaT cells proved that this cellular model is a valuable model in relation to metal speciation. Different oxidation state of metals shows different degree of toxicity: Pt(IV) is more toxic than Pt(II); Se(IV) is more toxic than Se(VI); Te(IV) is more toxic than Te(VI) and Cr(VI) than Cr(III) (Table 5.3).

Table 5.3- Comparison between the cytotoxicity induced by metal compounds with different oxidation state in HaCaT cells exposed to 100μM for 72h

Metal compound	Oxidation state	CFE ± SD (% of the control)
Control		100
$(NH_4)_2PtCl_4$	Pt(II)	58.4± 8.5
$(NH_4)_2PtCl_6$	Pt(IV)	0.0
Na_2SeO_3	Se(IV)	17.8± 5.3
Na_2SeO_4	Se(VI)	88.5± 16
Na_2TeO_3	Te(IV)	3.00± 5.5
Na_2TeO_4	Te(VI)	36.2± 10
$CrCl_3$	Cr(III)	88.9± 7.9
Ca_2CrO_4	Cr(VI)	0.0

Interestingly, Ag, Au, Cr, Cu, Hg, Pt and Se have been reported to induce allergic contact dermatitis, allergic contact urticaria and systemic contact allergy (Table 5.4), further proving the validity of HaCaT model for the study of potential sensitisation of metal compounds.

Table 5.4.- Multiple immunological effects of metals classified in Group 3 as drawn from the present study on HaCaT.

Element	ACU[a]	ACD[b]	SCA[c]
Ag	+	+	-
Au	+	+	-
Cr	+	+	+
Cu	+	+	+
Hg	+	+	+
Pb	+	+	+
Pt	+	+	-
Se	-	+	-

a: Allergic Contact Urticaria
b: Allergic Contact Dermatitis
c: Systemic Contact Allergy

PC12 cell line

Different systematic studies (screening tests) relative to the cytotoxic effect induced by 24 metal compounds have been performed on PC12 cells. In particular, the study has been performed on genetically engineered modified PC12 cell line. The cells were grown in the presence or absence of tetracycline. This approach is very interesting: after addition of tetracycline to the growth medium, PC12 cell lines expressing p- 53 under Tet- off control, provides a panel of cells with different sensitivities towards cell death, together with their specific neuronal cell characteristics (Stingele, 2000). This allows the possibility of performing mechanistic studies, testing the developmental and post developmental

neurotoxicities of a wide array of endogenous/ exogenous factors, and the screening for inhibition/ activation of cell death (Stingele, 2000). Thus, these cell lines provide an innovative, mechanistically relevant, and sensitive "in vitro" model for metal neurotoxicity studies, which could be considered for prevalidation and validation according to ECVAM's criteria (Balls and Fentem, 1997).

In the present study, the MTT reducing capacity of p- 53 cells grown in presence or absence of gene expression was determined after treatments with metal compounds. In particular, cytotoxic effects of different metal compounds have been tested under different experimental conditions: two different culture media (Tet- on and Tet- off), 24h and/ or 72h of exposure, and exposure of cells to a fixed dose of metal compounds. Firstly, a screening cytotoxicity test by MTT incorporation was carried out on 14 metal species in PC12 cells with two types of growth media (Tet- on and Tet- off), in relation to the expression of p- 53. The findings (Table 4.18) showed that the approach of "Tet- on medium" would seem more sensible in relation to the neurotoxic response of metals (Table 5.5). Then, the subsequent systematic screening test on 24 metal compounds has been carried out using Tet- on medium at two different exposure time (24 and 72h Table 4.12). The findings confirm again importance of the time of exposure on the neurotoxic response induced by metal exposure.

Table 5.5- Degree of neurotoxic effect induced by 100μM of metal compounds in PC12 (Tet- on medium) as a function of the time of exposure (24 or 72h).

Degree of cytotoxic effect	Metal species
No significant effect	Al, As (as $NaAsF_6$), DMA
72> 24h	Ag (I), As (as $NaAsO_2$, Cd, Cr, Pt, Se (IV), Te (VI)

Being Tet- on medium more sensible for the cells than Tet- off medium and being the cells generally more sensible to metal compounds when treated for 72h, a dose response study has been performed on the cytotoxic effect of $Al(NO_3)_3$, $NaAsO_2$, $NaAsF_6$, $HgCl_2$, CH_3HgCl, $MnCl_2$, $KMnO_4$, $(NH_4)_2PtCl_4$, $(NH_4)_2PtCl_6$, $SnCl_2$, Na_2TeO_3 and Na_2TeO_4.

A dose dependent cytotoxicity response was observed for $NaAsO_2$, $HgCl_2$, $(NH_4)_2PtCl_4$, Na_2TeO_3 and Na_2TeO_4. In all cases the interval of concentrations in which IC_{50} take place ranges from 0.1 to 100 μM(Table 5.6).

Table 5.6- Interval of concentrations in which IC_{50} take place.

Metal compound	IC_{50} (interval of concentration) (μM)
$NaAsO_2$	1.0- 3.0
Na_2TeO_4	5.0- 10
$HgCl_2$	10- 100
$(NH_4)_2PtCl_6$	0.1- 1.0
CH_3HgCl	0.1- 1.0
Na_2TeO_3	0.1- 1.0

No effect was observed for the salts: $Al(NO_3)_3$, $NaAsF_6$, $MnCl_2$, $KMnO_4$, $(NH_4)_2PtCl_6$, and $SnCl_2$.

Embryonic stem cells, D3

A study (Es Cell Differentiation Assay) relative to the effects induced by methyl mercury on the beating of Es D3 cells has been performed. It is known that methyl mercury crosses the palcenta and has proven to be a potent teratogen (Toshima et al.,1979). It induces a number of abnormalities, especially of the central nervous system (Iwata et al., 1973). Administration of methyl mercury compounds to pregnant mice induced brain and jaw defects, cleft palate and postbehavioral alterations. Methyl mercury is the cause of birth defects and neurological deficits in Minamata disease (Takeuchi, 1966). It has also been shown to be embryotoxic and teratogenic in golden hamsters (Harris et al., 1972; Hoskins and Hupp, 1978), rats and mice. In this preliminary study on the effect of CH_3HgCl on embryonic stem cells, D3 the aim was to identify at what differentiation stage(s) metal would act. The finding that cytotoxic effect of the metal compound was observed only during the first step of the differentiation (Table 4.26) is in agreement with the results obtained by Su (1976) who demonstrated a significant inhibition in cell proliferation of of the blstocyst stage embryo following the exposure of methyl mercury chloride while no effect was observed during the latest steps of the procedure.

BALB/3T3 cell line

The findings of the uptake of arsenic after exposure of cells to inorganic As (III) or As (V) species (Fig. 4.1) shows that the penetration of As into the cells occurs by an “active mechanism” which can be explained by the ability of inorganic As (III) or As (V) ions to interact with cellular components (Vahter and Marafante, 1988). On the contrary, the cellular uptake of As exposure to arsenobetaine and arsenocholine involves a “simple

diffusion” mechanism towards the penetration into cellular components since no binding with cellular components would occur (Sabbioni et al., 1991). This conclusions are in agreement with “in vivo” studies on the metabolic pathways of organoarsenic species which showed a very low capability of intracellular binding of As with macromolecules.

Unlike inorganic $NaAsO_2$ and $NaAsO_4$ arsenobetaine and arsenocholine failed to induce any significant cytotoxic effect in BALB/3T3 cell line giving the inorganic As species but not the organic ones obvious positive results in the morphological neoplastic transformation assay. These findings confirm that inorganic As compounds are biologically more active compared to organo arsenic species.

CONCLUSIONS AND SOME PRIORITIES FOR FUTURE RESEARCH

The work of he present thesis confirms how the toxicological screening is one of the most important application of “in vitro” toxicity testing, giving a general indication of the intrinsic toxic potential of test chemicals, in our case metal compounds. In particular, we have adopted a strategy based on the determination of cytotoxicity induced in HaCaT and PC12 cell lines by a fixed- dose exposure of metal compounds. This, in order to identify the most cytotoxic metal species, and then setting dose- effect relationships on these latter metals to establish a dosage compatible with cell injury, to identify the best experimental conditions for subsequent mechanistically- based investigations, and to identify metal compounds of priority in future prevalidation/ validation studies. In this context, the findings obtained on immortalised human keratinocytes (HaCaT) and rat pheochromocytoma (PC12) cell line (Tables 4.7 and 4.12) suggest that the 10 metal compounds which are highly toxic to PC12 cells are also toxic to HaCaT cells (Table 5.7), although in this latter case other 8 metal species were found to be strongly cytotoxic. Thus, these metals would have the highest priorities in subsequent studies of their toxic mechanism.

Table 5.7- Metal species with high priority in subsequent dose- effect relationships studies on the basis of their highest toxicity as identified by screening studies (Tables 4.5, 4.6, 4.7, 4.11 and 4.12).

Cellular model considered	
HaCaT	PC12
Ag (I), Au (III), Cd (II), Cr (VI), Cu (II), Ga (III), Hg (II), HgMe (II), Mn (II), Mn (VII), Pb (II), Pt (VI), Se (IV), Te (VI), Te (IV), V (IV), V (V).	Ag (I), As (III), Cd (II), Cr (VI), Hg (II), HgMe (II), Mn (VII), Pt (VI), Te (IV), V (V).

The investigations here carried out have also proved how “in vitro” cellular models give positive responses in relation to the influence of speciation (different chemical forms of an individual element). The cases of Pd, Pt, Hg, Mn, Se and Te (studies on HaCaT) of Hg, Mn, Pt, and Te(by PC12) and of As (by BALB/3T3) show clearly how the chemical form of the metal affects the cytotoxic and morphological transformation responses.

The results obtained on arsenic compounds (Figures 4.1, 4.2 and Table 4.23) confirm also the great potential of the “in vitro” toxicity testing methods in combination with the use of nuclear and radioanalytical techniques in investigations relating metabolism and toxicity of metal compounds in mammalian cells (Sabbioni and Balls, 1995). In particular, the availability of carrier- free radiotracers such as ^{74} As produced at the cyclotron and the rapid, simple and high sensitivity detection and measurement of its characteristic gamma radiations allowed the study of the uptake of the metal by cells (Figures 4.1 and 4.2), a fundamental parameter in mechanistically- based “in vitro” metal toxicology research to develop uptake- effect relationships (IMETOX) (Sabbioni and Balls, 1995). However in the case of arsenobetaine and arsenocholine the radiolabelling by ^{74} As radiotracer was not

possible. In this case, neutron activation analysis with its high specificity, accuracy and sensitivity for arsenic played an essential role in making possible the study of the uptake of such compounds by the cells (Figures 4.1 and 4.2).

These findings must be intended as a contribution to the information concerning a general view of metal toxicology, encouraging the use of test battery in the context of “in vitro” integrating testing strategy (Barratt et al., 1995). The present research is, however, far to be exhaustive but it must be considered a starting point for future investigations on metabolic patterns of metal compounds that have been identified of “high priority” by our screening toxicity testing.In spite of the possibility of investigating basal cell functions by cell cultures, few systematic studies on the interactions of metal compounds and cells at the basal cytotoxic concentration level have been performed. This implies that only for few metal compounds mechanistic interpretation of the cytotoxicity is available as essential aspect to give a sound scientific basis to the evaluation of human risk associated with trace metal exposure. Thus, our screening of metal toxicity by cell cultures must be seen as a temporary solution of the problem of metal toxicity, rather than a goal. The ultimate goal is to acquire a sound knowledge of the processes involved in cytotoxicity as well as of systemic toxicity of tested metal species.

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