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"In Vitro Rodent Models as Alternative Methods in Assessing Cytotoxicity and Carcinogenic Potential of Metal Compounds"

Report of the Research Activity Presented by ***Francesca Mazzotti***

to Obtain the Title of PhD

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With all these people I would like to share the following passages.

<< Another questioner has asked: "What is there more to peace than non-war?"

Why not observe our own life? Is there peace in your life only when you are not fighting with someone else, quarrelling with someone else? The absence of verbal quarrel, psychological clash, does it constitute peace in your life? Has peace any positive and constructive content as far as your daily living is concerned?

It seems to me that peace is related to the awareness of what is the aim, the objective, the purpose of life. As long as living is a means to an end and not an end in itself and by itself, there cannot be peace in the life of an individual.

Going to a school or a university is a means to acquire a degree, a diploma. That is a means for acquiring a job. A job is a means to acquire money. Money is a means to purchase social security and so on and so on. We are not concerned with living, the quality expressed in the movement of our relationship with nature, with ourselves, with fellow non-human species, with fellow human beings and so on. The quality, the essence of life is neglected completely and living becomes only a chain-acquisitive process, like chain-smoking, it is chain-acquiring. Go on acquiring; acquire, own, possess, protect and die in the end feeling content that you had acquired so much and you had so much wealth and you are leaving behind so much property. Do you see? Living becomes a means to an end. Not for the joy of it, not because life is something sacred.

Life is for living. The purpose of life cannot be outside living. The act of living cannot be a means to an end, to please some god or goddess, to acquire money, prestige, security, etc. But the act of living is the worship of the divine. It is the only way one can express gratitude, gratefulness to the cosmic life of which one is born and in which one is living and moving.

It seems to me that there cannot be peace; you may be a decent human being and you have cultivated the art of controlling your emotions, therefore you do not have quarrels, clashes, battles with other human beings, but controlling, curbing, restraining is not enough.

Peace requires a different perspective of life, it requires purification of perception. Life not as a means to an end, but life as an end in itself. Life itself is divine. Life is divinity. It is something sacred. And reverence for life is the perfume of religiosity. There is no other religion, but reverence for life that is self-generated and self-sustained. There

cannot be peace unless there is this radically qualitatively different perspective of life and therefore a different approach to life and a different attitude to human issues, challenges, problems. Peace is not only non-war or non-aggression. It is moving from a fragmentary partial or compartmental perspective of life to a holistic perspective of life. It is moving from the dimension of psychology of confrontation to psychology of cooperation >> *(Vimala Thakar: "Radical Peace")*

<< *Therefore I tell you, do not worry about your life, what you will eat or drink; or about your body, what you will wear. Is not life more important than food, and the body more important than clothes? Look at the birds of the air; they do not sow or reap or store away in barns, and yet your heavenly Father feeds them. Are you not much more valuable than they? Who of you by worrying can add a single hour to his life?*
And why do you worry about clothes? See how the lilies of the field grow. They do not labour or spin. Yet I tell you that not even Solomon in all his splendour was dressed like one of these. If that is how God clothes the grass of the field, which is here today and tomorrow is thrown into the fire, will he not much more clothe you, O you of little faith? So do not worry, saying, 'What shall we drink?' or 'What shall we wear?' For the pagans run after all these things, and your heavenly Father knows that you need them. But seek first his kingdom and his righteousness, and all these things will be given to you as well. Therefore do not worry about tomorrow, for tomorrow will worry about itself. Each day has enough trouble of its own >> *(Matthew 6, 25-34)*

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INTRODUCTION

Cell Transformation Assays as Predictors of Human Carcinogenicity

It is well established that the carcinogenic process can be modelled *in vitro* on the basis of morphological cell transformation *(Combes, R. et al., 1999)*. Several cell transformation assays based on immortalised or non-immortalised rodent cell systems were developed a long time ago, and have been shown to be capable to detect some well-knowing animal and human carcinogens, as well as tumour promoters. In fact, many of the stages of carcinogenesis, as well as the role of oncogenes, have been established as a result of *in vitro* studies on cell transformation *(Worth A.P. and Balls M., 2002)*.

Actually, the likelihood that a particular *in vivo* toxicological assay is replaced by an *in vitro* method is dependent on factors such as:

a) mechanistic understanding of toxicological process *(Purchase I.F.H., 1992*). For some toxicological events, the mechanism of toxicity is only partially understood and unknown, e.g. the role of somatic mutation in the development of chemically-induced cancer, and the influence of certain physicochemical properties on the stratum corneum, which results in skin corrosion. Therefore, it is likely that an *in vitro* method based on the same mechanism can be developed satisfactorily. However, for the majority of toxicological endpoints, the knowledge of the underlying mechanism is available only for a few chemical compounds, which precludes its extrapolation to same families of chemicals. This is particularly relevant for endpoints, such as lethality, which at first sight appear relatively simple. The hypothesis that chemicals cause basal cytotoxicity is beguilingly simple. It does not take into account the plethora of pathological processes, including necrosis, apoptosis, hyperplasia, metaplasia and fibrosis, which are observed in toxicological studies, of the complex interplay of hormones and cytokines in cellular control, which so often contribute to toxicological events.

b) complexity of endpoints *(Purchase I.F.H., 1996*). Skin corrosion is an

example of an endpoint, which is relatively simple to observe and therefore to mimic *in vitro*. However, the majority of toxicological studies, such as chronic studies, have a multiple of endpoints under observation, including pathological, haemathological and clinical signs. It becomes more difficult to conceive *in vitro* methods, which can mimic these complex events.

c) nature of exposure *(Purchase I.F.H., 1996)*. For some toxicological endpoints, for example the induction of cirrhosis or emphysema, repeated injury to the organ is a prerequisite for the development of the lesion. In many cases, dosing for 6-12 months is required before the lesion becomes manifest. Once again, it is difficult to conceive *in vitro* methods, which can mimic these conditions (chronic exposure), particularly in a quantitative sense.

In evaluating the future development of *in vitro* methods, it is concluded that methods which are "easiest to develop" have provided the most encouraging results *(Combes, R. et al., 1999)*. The "most difficult methods" will be those, which use repeated exposure inducing many effects evaluated by toxicological endpoints and explaining the mechanisms of such effects. It is difficult to predict when suitable validated *in vitro* methods will be available to replace the well-established animal methods, but it is likely to be many years hence. In this context, ECVAM (the European Centre for the Validation of Alternative Methods) has been established to shorten the time of this process.

IMETOX (*In vitro* MEtal TOXicology project to which the present work belongs) is one of ECVAM's researches to seek consensus on ways of increasing the use of mammalian cell transformation assays, especially in human cell systems, for fundamental and applied studies in carcinogenesis, and for the regulatory testing of carcinogens. This is endorsed by the fundamental belief that the further development, evaluation, routine use and eventual regulatory acceptance of cell transformation assays, in conjunction with other toxicity information, would improve the overall process of safety and risk assessment of carcinogenicity for the protection of human health.

At present there are several reasons for development and use of cell transformation assays to predict carcinogenicity such as technical skills, lack of characterisation and standardisation, and the fact that the relevance and reliability of the assays have not been established in independent studies in view of the validation for regulatory purpose. Consequently, an important question to address is the extent to

which information from cell transformation assays can be used to reduce the uncertainty that human exposure to a chemical under a given set of conditions will pose a carcinogenic risk. It is recognised that this risk will vary according to chemicals and conditions concerned. It is recommended that there should be a case-by-case approach to the regulatory use of cell transformation data, rather than a single strategy such as one, which limits this use to compound selection *(Combes, R. et al., 1999)*.

The European Union Chemical Policy

A Relevant Opportunity to Speed the Development of Alternative (Non-animal) Methods

The interrelationship among toxicological impact of chemical substances, human health, and risk assessment, is becoming a problematic issue for economical and political point of view. Since in the early days of the European Community it was recognised the need to set standards to protect the Community's environment and consumers in order to ensure the free circulation of goods among the Member States. For this reason, the first Community environmental legislation dealt with products, amongst them dangerous chemicals. Nevertheless, in recent years, many drawbacks of the current system have been identified. One of the major problems examined concerns the lack of knowledge about the potential danger of many existing substances, which represent about 99% of the total volume of chemicals on the market. This is due to a too slow process of risk assessment, with only a handful of existing chemicals being assessed. Moreover, resources are concentrated too much on the assessment of new chemicals, which make up only about 1% of the total volume of substances on the market *(Anon, 2001a)*.

With a view to overcome the weaknesses of the current system of the EU chemicals legislation, on 13th February, 2001, the European Commission adopted a White Paper setting out the strategy for a future Community Policy for Chemicals *(Anon, 2001b)*. The White Paper strives to balance the essential need to protect human health and environment with the requirement to maintain and enhance innovation and the competitiveness of the EU chemical industry. The proposed EU Chemicals Strategy

entails a new division of labour and responsibility between industry, Member States and the European Community. The "burden of proof" is hereinafter shifted from authorities to industry under a clear set of legal obligations with a view to ensuring a high level of protection of human health and the environment through making industry responsible for the safety of its products. Doing so, this new system will encourage substitution of dangerous by less dangerous substances where suitable alternatives exist, and will provide incentives for technical innovation and the development of safer chemicals *(Anon, 2001c)*.

The White Paper proposes to harmonise the testing requirements for new and existing substances, by introducing a new system for the Registration, Evaluation and Authorisation of new and existing chemical substances, known as the REACH system *(Worth A.P. and Balls M., 2002)*. It is estimated that implementation of the new chemicals policy will result in the need for the further assessment of up 30,100 existing chemicals, which are currently marketed in volumes greater than 1 tonne per year (tpa), and for which essential human health and ecotoxicological data are lacking. The proposed schedule for registration depends on the production/importation volume of the chemical (Table I).

Table I: The stepwise approach

(Worth A.P. and Balls M.. 2002)

Number of substances	Volume (tonnes per annum)	Deadline for registration
2600	>1000	End of 2005
2900	100-1000	End of 2008
4600	10-100	End of 2012
20,000	1-10	End of 2012

The amount of testing required will be triggered partly the production/importation volume (Table II). It is proposed that chemicals with volumes in the range 1-10 tpa should be tested with *in vitro* methods alone. This means that a set of appropriate alternative methods should be available to permit the registration of such substances. It should also be noted that chemicals produced/imported in amounts higher than 10 tpa are not necessarily excluded from *in vitro* testing. In addition, the testing of chemicals of particular concern, such as CMRs (carcinogenic, mutagenic and reprotoxic substances) and POPs (persistent organic pollutants), may be required, even if they are marketed in volumes of less than 1 tpa *(Anon, 2001d; Worth A.P. and Balls M., 2002)*.

Table II: The tiered approach [a]

Volume (tonnes per annum)	Testing required [b]
<1	Self-responsibility of industry
1-10	Generally *in vitro* methods
10-100	Base-set testing
100-1000	Base-set testing + Level 1 testing, i.e. "substance-tailored testing for long-term effects"
>1000	Base-set testing + Level 2 testing, i.e. "additional substance-tailored testing for long-term effects"

a: Worth A.P. and Balls M., 2002.
b: Base set: Acute toxicity – Repeated dose toxicity – Mutagenicity (Ames)
Level 1 & Level 2 : (Sub-) Chronic exposure – Reproductive toxicity – Carcinogenicity

To maximise the number of alternative tests available and suitable for use in the REACH system, emphasis should be placed on the development and validation of alternative tests that are already considered to be promising. This because validated and accepted tests are likely to be required by 2008, in order to meet the proposed deadline of 2012 for the testing of the 20,000 existing chemicals produced in the range of 1-10 tpa (Table I). Such a strategy represents the only feasible way for industry. In fact, the reason why only a few of the huge amount of the existing chemicals have been subjected to toxicology testing and evaluated to any satisfactory extent, is the impossibility to raise the necessary resources to cover the costs of a testing program running animal tests on all these chemicals. If simplified methods are available, the cost-benefit analysis for toxicological testing could be improved, thereby making it possible for decision to be made about which chemicals needed to be tested further with the more time-consuming *in vivo* methods *(Walum E. et al., 1990)*.

The Evolution in the Use of Animal Experiments

The use of animal experimentation is a complicated issue. For example, the animal models (e.g. monkeys and baboons) are considered the most useful for the extrapolation of the results to humans, but ethical reasons impose to avoid their use *(Balls M. et al., 1995)*. In addition, due to differences among species, the use of animal models will never provide results that are perfectly relevant to humans. Then, the animal welfare case against animal experimentation is very strong. Nevertheless, an equally convincing argument can be made that it would be improper to deny the benefits that animal experimentation can offer, both to science and to the sick and suffering,

until suitable alternatives to animal procedures can be found.

Animals are used in experiments to predict potential adverse effects of chemical substances in humans, particularly *(FRAME, 2002a)*: a) to improve our basic knowledge of the complicated biological systems that keep humans and other animals alive and well, and to understand the pathological effects on these systems; b) to devise new ways of diagnosing diseases; c) to develop new treatments for diseases, including better medicines and surgical techniques; d) to produce useful biological products that can be used in preventing and treating diseases (e.g. vaccines, insulin for diabetics); e) to test the safety of products, such as medicines, vaccines, household products, cosmetics, toiletries, agriculture chemicals, substances used in industry, and food additives to ensure that they are safe to those using, making and handling them.

There are three possible ways in which the situation could change *(FRAME, 2002a)*:

a) Things could continue as now, or there could even be an increase in the number of animal experiments. Presently, we have no effective treatments for many serious and life-threatening diseases such as heart disease, and many types of cancers, AIDS and new-variant CJD. Research involving animals may lead to breakthroughs in these areas.

b) Experiments and tests involving the use of laboratory animals could be banned completely. However, an immediate and total ban of the use of animals would have an acceptable negative impact on human health, e.g. a great deal of basic medical research and production of certain vaccines would be stopped, no new medicines would be developed. Thus, health of the general population, occupational workers and patients would be jeopardised.

c) There could be a progressive move to reduce, refine and replace the use of animals in experiments. This is called the 3Rs (Reduction, Refinement, Replacement) approach and would appear to be the most sensible way forward *(Russel W.M.S. and Burch R.L., 1959)*. It offers the chance for scientists and animal welfare activists to work together.

Non-animal Alternatives and the Validation Process: Which Duties of the European Centre for the Validation of Alternative Methods?

The application of an integrated approach to *in vitro* toxicity testing should involve the simultaneous consideration of all the Three Rs, as well as the integrated and non-competitive use of a variety of animal and non-animal tests that are, or will increasingly become, available. In this context, ECVAM promotes a dialogue between legislators, industries, biomedical scientists, consumer organisations and animal welfare groups, with the aim to develop and coordinate the validation of alternative test methods at the EU level as well as their international recognition *(Balls M., 2001)*.

This is in accordance with the 1991 Commission Communication on the basis of which the European Commission established ECVAM (Figure 1) in response to Article 23 of Directive 86/609/EEC *(Anon, 1986)*. The establishment of ECVAM has reflected the general commitment of the EU to the Three Rs approach (Table III) that provides a strategy for a rational and stepwise approach to minimising animal use and the suffering caused by this use, without compromising the quality of the scientific work being done, while having, as the ultimate aim, total replacement of animal models with non-animal alternatives *(Fentem J.H. and Balls M., 1997)*.

Figure 1: ECVAM's logo

Table III: The Three Rs **(FRAME, 2002b)**

Reduction	*The concept of reduction alternatives covers any strategy that will result in less animals being used to obtain the same amount of information obtained per animal and thus limiting or avoiding the subsequent use of additional animals. There are several possible approaches that can serve to reduce the use of animals: an example is the Local Lymph Node Assay (LLNA). Appropriate experimental design and appropriate analysis of the resulting data, with due consideration to statistical principles, can increase the precision of the data and at the same time enable fewer animals to be used for the generation of these data.*
Refinement	*The term refinement signifies the modification of any procedures that operate from the time a laboratory animal is born until its death, so as to minimise the pain and distress experienced by the animal, and to enhance its well being. Giving due consideration to issue of animal welfare is not only important from the viewpoint of ethics, it is also a matter of good science. The experience of pain and other stress is likely to result in physiological changes, which may increase the variability of experimental results. Therefore, it is in the interest of scientists to ensure that conditions in the animal house are the best possible. The use of infrared measurement photography is an example of a refinement alternative.*
Replacement	*Any experimental system, which does not entail the use of a whole, living animal is considered to be a replacement alternative. Some of these are relative replacements, as they still entail the humane killing of an animal for the purpose of obtaining cells, tissues or organs for subsequent in vitro studies. Others are absolute alternatives, which do not require any biological material derived from a fully developed vertebrate, non-human animal. In some cases, they will complement animal experiments and serve to reduce the overall number of animals used in the whole project. A major impetus to the development of replacement alternatives is provided by the rapidity with which the pharmaceutical industry can now candidate drug compounds. Replacement alternatives can be divided into six categories: information; computer-based system; physico-chemical techniques; the use of lower organisms and embryo stages; human studies; and cell, tissue and organ cultures.*

The focal point of ECVAM is the validation of alternative testing methods. This is the process whereby the reliability and relevance of a procedure are established for a particular purpose *(Balls M. and Fentem J.H., 1999)*. The relevance of a procedure refers to the scientific value and the practical usefulness of the results it provides, whereas reliability is concerned with the reproducibility of these results within and between laboratories and over time, in relation to a clearly defined and specific purpose *(Balls M. et al., 1990)*.

The ECVAM scientific validation process consists of different stages: prevalidation and formal validation (Table IV). The aim of the prevalidation stage between test development and formal validation is to improve the efficiency, speed of the validation process, and to maximise the likelihood of a successful outcome. This to ensure that a test protocol has been optimised in an experienced laboratory, which developed the test, and that the optimised protocol is transferable to a third laboratory.

A formal validation study is a larger interlaboratory study, performed under blind conditions, designed to obtain a more definitive assessment of relevance and reliability and in which a larger number of chemicals compared to prevalidation are tested *(Worth A.P. and Balls M., 2001)*.

After a formal validation study has been completed, the management team should make a report, which is published in the peer review literature. This is accompanied by an independent evaluation of the goals of the study, its design, management and conduct, and its outcome, by one or more appropriate agencies, such as independent representatives of the sponsors of the study. This evaluation can lead to scientific and industrial acceptance of the test in order to draft a proposed regulatory guideline, and to mark the beginning of the progression of the test towards regulatory acceptance and application. Test validation is therefore a formal, political and regulatory driven process, which nevertheless ought to be less scientifically defensible *(Balls M. and Fentem J.H., 1999)*.

In this context, the mechanistic approach in development toxicity testing is mandatory. Unfortunately, most existing *in vitro* tests are not developed enough to generate reliable data and lack of a sufficient mechanistic knowledge. There is a great need to develop mechanistically based *in vitro* toxicity tests to be consider in the regulatory legislation on chemicals *(Bruner L. et al., 1996; Purchase I.F.H., 1996)*.

Table IV: Stages in the evolution of new tests

1. *Test development* (laboratory of origin)

   Purpose of the test
   Need for the test
   Derivation of the method
   Application to appropriate chemicals
   Case for inclusion in a validation study
   Production of a protocol
   Development of a prediction model

2. *Prevalidation* (informal interlaboratory study)

   Optimisation of the test protocol
   Assessment of its interlaboratory transferability
   Optimisation of the prediction model

3. *Validation* (formal interlaboratory study, including a blind trial)

   Two phases: preliminary phase (training set of chemicals)
   definitive phase (test set of chemicals)
   Main stages: study design
   selection of tests
   selection of laboratories
   selection, distribution and testing of chemicals
   data collection and analysis
   assessment of performance of test
   assessment of applicability of prediction model

4. *Independent assessment* (of study and proposals)

5. *Progression toward regulatory acceptance*

OBJECTIVES OF THE PRESENT STUDY

♦ **Check of chemical purity** of metal compounds used in cytotoxicity and transformation assays in order to avoid possible artefacts about the biological response.

♦ **Standardisation and optimisation of Balb/3T3 cell transformation assay** (mouse embryo fibroblasts) in order to assess intralaboratory reproducibility and transferability of test protocol among different laboratories in view of future prevalidation/validation studies. The study includes a quality check of Balb/3T3 cells, culture medium, its pH and serum as well as the use of two endpoints (Colony Forming Efficiency (CFE) and Neutral Red Uptake (NRU)) for the determination of cytotoxicity of Balb/3T3 cells exposed to metal compounds.

♦ **Determination of cytotoxicity and carcinogenic potential** of inorganic and organometallic compounds **by the Balb/3T3 assay**. The study is based on a "four steps approach": *i*) determination of cytotoxicity at a fixed concentration exposure (100 μM, 65 individual metal compounds). This allows the selection of metal compounds to which priority is given for subsequent investigations; *ii*) setting of dose-effect relationships for metal compounds identified as first priority in step (*i*), in order to establish the IC_{50} values and a suitable exposure range of concentrations to be used in the subsequent step; *iii*) determination of concurrent cytotoxicity and morphological transformation of selected metal compounds from step (*ii*); *iv*) mechanistic studies on metal species identified transforming from step (*iii*).

♦ Analysis of the ability of selected metal compounds (As, Cr, Pt) to induce **apoptosis in the Balb/3T3 cells**, as an initial mechanistic approach.

♦ A study of basal cytotoxicity induced by selected Pt- and As-compounds on immortalised human keratinocytes **HaCaT cell line** in view of its use as a potential cell transformation assay of human origin.

♦ Evaluation of reproducibility and transferability of the test protocol of **Syrian Hamster Embryo (SHE)** cell transformation assay to be compared to Balb/3T3 assay.

♦ **Cellular uptake, intracellular repartition and speciation** in culture medium of As-, Cr- and Pt-compounds using radioanalytical methods (^{73}As and ^{51}Cr radiotracers) and advanced spectrochemical techniques (ICP-MS and NMR) as initial metabolic studies for the interpretation of the induced metal toxicity.

LITERATURE SURVEY

1 Carcinogenesis and Cell Transformation

Carcinogenesis is a multistep process, which involves sequential genetic alterations in a single target cell, which cause subtle alterations in growth control and culminate in cells that are able to form malignant tumours *(Barrett J.C., 1993; Maronpot R.R., 1991)* (Figure 1.1).

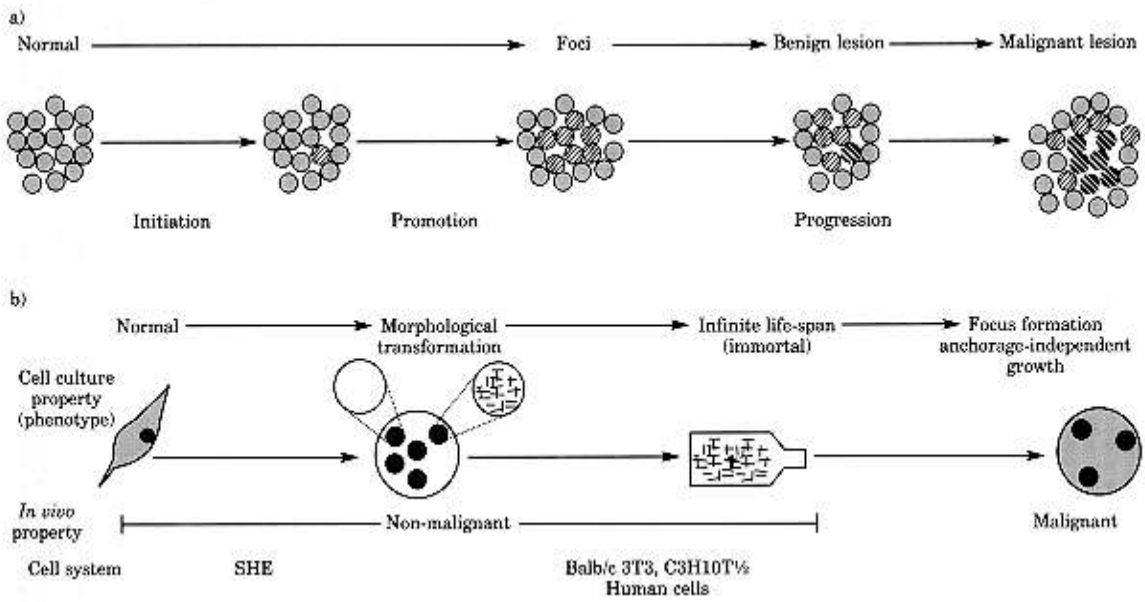

Figure 1.1: Representation of a) carcinogenesis in vivo and b) cell transformation in vitro **(Combes R.D. et al., 1999)**

Genetic changes can result from spontaneous or carcinogen-induced alterations in DNA. Non-genotoxic mechanisms, that are at least initially independent of direct DNA damage, can play a role in carcinogenesis *(Combes R.D., 1997)*. Research to elucidate the mechanisms of carcinogenesis has involved experimental animal models, *in vitro* cellular systems as well as clinical and epidemiological studies. In particular, the study of carcinogenesis was greatly facilitated by the discovery of *in vitro* morphological transformation of mammalian cells in culture some 35 years ago *(Berwald Y. and Sachs L., 1965; Meyer A.L., 1983)*. The phenomenon of morphological cell transformation

involves changes in the behaviour and growth control of cultured cells, characterised by one or more of the followings: alterations of cellular morphology; disorganised patterns of colony growth; and acquisition of anchorage-independent growth (Figure 1.1) *(Barrett J.C. and Fletcher W.F., 1987; Yuspa S.H. and Poirier M.C., 1988)*. Such effects result from changes in the expression of oncogenes and/or tumour suppressor genes.

Cell transformation has been defined as the induction of certain phenotypic alterations in cultured cells that are characteristic of tumorigenic cells. These phenotypic alterations can be induced by exposing normal cells to carcinogens, or by expressing activated oncogenes in such cells. Transformed cells that have acquired all the characteristics of malignant cells have the ability to form invasive tumours in susceptible animals *(Barrett J.C et al., 1986)*.

The conventional approach to carcinogenicity testing is the lifetime rodent bioassay (rats and mice of both sexes) with pathological analysis of tissues. This test is used to detect complete carcinogens, as well as tumour promoters and co-carcinogens. However, the rodent bioassay is time-consuming, labour-intensive and costly. In addition, rat and mouse data do not correlate well, and extrapolating the information to humans is problematic *(Combes R.D., 1997; Gottmann E. et al., 2001)*. This is a serious problem if one considers that chemical exposure can have two different impacts on human health. In fact, genotoxic chemicals are likely to exhibit trans-species carcinogenicity, often in both sexes, at intermediate dose-levels, and this will not necessarily be restricted to one target tissue *(Ashby J. and Tennant R.W., 1991)*. These chemicals are the so-called "genotoxic carcinogens". However, an increasing number of chemicals lacking any genotoxicity are proving to exhibit carcinogenicity, and this activity is often manifested in one species, one sex and even in one specific tissue, and usually only at high dose-levels *(Choy W.N., 2001)*. These chemicals are so-called "non-genotoxic carcinogens". The mechanisms involved in non-genotoxic carcinogenesis are less well characterised than in genotoxic carcinogenesis. As a consequence, considerably less information is available for generating useful rules for predicting this type of activity *(Combes R.D., 2000)*. At present, chemicals that prove to be non-genotoxic *in vitro* and *in vivo* may still be subjected to a rodent bioassay, depending on their use, and if a high level of human exposure is anticipated. However, the cost involved in rodent bioassays is not suitable for dealing with large numbers of chemicals.

Cell transformation assays are sensitive to a wide range of both genotoxic and non-genotoxic carcinogens, especially those that act by way of epigenetic effects and as tumour promoters, and affect cellular proliferation. Actually, the main endpoints of cell transformation (focus formation and the acquisition of the ability to grow in soft agar) result from loss of contact inhibition and of anchorage-dependence, respectively. The relationship between these cellular endpoints, which can be measured in cell culture and *in vivo* carcinogenesis and malignancy, can be demonstrated by malignant tumour formation after the transformed cells are injected into animals *(Mareel M.M et al., 1979; Smolle J. et al., 1992)*. Since is difficult to model later stages of carcinogenesis, *in vivo* experiments on cancer cell invasiveness and metastasis are limited. Therefore, it is essential to implement *in vitro* studies. Good examples are the three-dimensional *in vitro* invasiveness assays *(DeVaney T.T. et al., 1997; Fusenig N.E. and Boukamp P., 1998)*.

1.1 Rodent Cell Transformation Assays

Cell transformation models have been extensively used for studying the cellular and molecular basis of the neoplastic process, the mechanisms of chemical carcinogenesis and the screening of chemicals for their carcinogenic potential. In case of rodent cell lines, transformation assays involve either finite life-span cells (Syrian Hamster Embryo (SHE) cells) or immortalised fibroblast cell lines (Balb/c3T3 and C3H/10T½ cells). These three transformation assays have significant historical databases and their use in carcinogen assessment has been subject of extensive studies and reviews. The results deriving from these assays for predicting chemical carcinogenicity show

Table 1.I: In vitro rodent cell transformation assays

Cells	Cell line / Primary cells	Two-stage assay	Test duration (weeks)	Marker	Origin
BALB/3T3 A31-1-1	Cell line	+	5	Focus	Mouse
C3H10T½	Cell line	+	6	Focus	Mouse
SHE	Primary cells	–	1	Colonies	Syrian hamster

high predictive value for the outcome of rodent bioassays and for predicting human carcinogens (Table 1.I).

When used in conjunction with information from other studies (structure-activity analysis of genetic toxicity, short-term studies *in vivo* and information on mechanisms of action) these tests are believed to allow accurate predictions of carcinogenic activity and the probable mode of carcinogenic action *(LeBoeuf R.A. et al., 1999)*. Moreover, the discrimination whether the transformation activity of a chemical is due to a genotoxic or a non-genotoxic mechanism is only one of the additional information provided in interpreting transformation data. Actually, cell transformation can be assessed simultaneously in the same cultures with other endpoints, such as gene mutation, sister-chromatid damage, micronucleus induction and aneuploidy *(Fritzenschaf H. et al., 1993; Landolph J.R., 1985; Tsutsui T. et al., 1997)*.

While cell transformation assays have been recognised as potentially useful for identifying carcinogens, experimental parameters that result in interlaboratory variation have affected their widespread use. Early studies showed that cell proliferation and transformation frequencies are influenced by several parameters, including pH, nature of serum, cell culture method, cell passage number, cell seeding density, mode of isolation and handling of cells (especially in the case of the SHE cell assay) *(Landolph J.R., 1985; Lubet R.A. et al., 1986; Schechtman L.M., 1985)*. Other criteria in optimising protocols include treatment period, cell division time required for fixation and expression of the transformation event, phenotypic properties of transformed colonies, *in vivo* tumorigenicity of transformed cells, and responsiveness of cells to distinguish carcinogens and non-carcinogens.

1.2 Balb/c3T3 Cell Transformation Assay

The Balb/c3T3 mouse cell transformation assay has been extensively investigated *(Matthews E.J., 1993a; Matthews E.J., 1993b; Matthews E.J., 1993c; Matthews E.J. et al., 1993a; Matthews E.J. et al., 1993b; Swierenga S.H.H. and Yamasaki H., 1992)*. The A31-1-13 and A31-1-1 subclones have been isolated from the parent clone and still represent the best-optimised subclones used in transformation assays. The Balb/c3T3 cell transformation system is based on a spontaneously immortalised cell line initially

derived from a mouse embryo by repeated cell passages *(Aaronson S.A. and Todaro G.J., 1968a,b)* and subsequently subcloned to generate the line, A31-1, that is typically used in this assay *(Kakunaga T., 1973)*. The Balb/c3T3 clone A31 cell line is contact inhibited and grows at high dilution showing a low saturation density, 50-60% of plating efficiency, hypotetraploydia with telocentric and acrocentric chromosomes. They can be transformed in tissue culture by oncogenic DNA SV40 virus and murine sarcoma virus. Their growth is on a monolayer showing a fibroblast-like morphology. The split period is about 16 hours and the cell division control is density-dependent. Even though this cell line shows some specific characteristics of a transformed cell line (heteroploydia, infinite life span, high cloning efficiency, altered morphology in comparison with the primary culture), it lacks of anchorage-independent growth and tumour formation when inoculated in nude mice (two of the critical markers for transformation). The spontaneous transformation frequency is low (0.71×10^{-6} for type III foci), while the chemically induced transformation frequency is depending on concentrations of carcinogens, duration of the treatment and cell density *(Aaronson S.A. and Todaro G.J., 1968a; DiPaolo J.A. et al., 1972; Hei T.K. et al., 1994; Kakunaga T., 1973)*.

The Balb/c3T3 assay provided relevant information concerning intercellular communication in the context of the mechanism of action of initiating and promoting agents *(Yamasaki H. and Enomoto T., 1985; Yamasaki H. et al., 1992)* as well as in relation to the concept of the control of transformed cells by neighbouring normal cells *(Bauer G., 1996; Sakamoto Y. et al., 1999; Yamasaki H. et al., 1987)*. Moreover, the chemically induced transformation in Balb/c3T3 cells established the relationship between *in vitro* transformation and cytotoxicity, carcinogenicity and genotoxicity, showing a high specificity and sensitivity for detection of cytotoxic and non-cytotoxic carcinogens and exclusion of non-carcinogens *(Matthews E.J., 1990)*.

Major modifications to the standard transformation assay with Balb/c3T3 cells included changes of exposure and post-exposure conditions for highly cytotoxic chemicals, and addition of a variety of forms of endogenous metabolic activation *(Combes R. et al., 1999)*. However, there are still a number of drawbacks, notably: a) some 4-6 weeks of cell culture are required for the development of unequivocally transformed foci; b) the frequency of transformation is relatively low; c) the assay is labour-intensive.

A new protocol has been developed to address some of these problems, and especially to shorten the length of the assay (this is currently the subject of an extensive validation study being conducted in Japan) *(Tsuchiya T. and Umeda M., 1995)*. This protocol involves clone A31-1-1 cells and a modified medium, in conjunction with a new treatment regimen. This should result in a substantial improvement in transformation frequency, in the formation of clearly transformed foci at an earlier stage of incubation, and in enhanced responsiveness of the assay to several carcinogens, as well as another protocol suitable for investigating promoting activity *(Tsuchiya T. and Umeda M., 1997)*.

1.3 Syrian Hamster Embryo Cell Transformation Assay

The Syrian Hamster Embryo (SHE) *in vitro* cell transformation is a neoplastic process that proceeds through several identifiable stages including *in vitro* morphological transformation, acquisition of immortality, acquisition of tumorigenicity and tumour-derived cells *(Isfort R.J. et al., 1994)*. SHE cells are primary diploid rodent cells with metabolising capacities and low spontaneous transformation frequency in contrast to established cell lines. In particular, the SHE cell transformation assay does not require the use of an exogenous source of metabolic activation, contrary to the most *in vitro* tests *(Pienta R.J., 1981; Pienta R.J. et al., 1977)*.

SHE cell transformation assay represents one of the earliest assays which used primary cells *(Berwald Y. and Sachs L., 1965; DiPaolo J.A., 1980; Lubet R.A. et al., 1986; Pienta R.J. et al., 1977)* and which considered the formation of transformed colonies as endpoint. The cells employed are used a few passages after the isolation of mixed populations of embryonic cells which are at various stages of differentiation and hence providing a broad spectrum of cellular targets for the neoplastic response. SHE cells have a limited life span in culture, and rarely become tumorigenic, unless exposed to a carcinogen. The theoretical advantage of using finite life span cells is that the individual changes that the cells must undergo to become malignant can be studied. In practice, however, quantitative tests have usually been developed for one step in the carcinogenic process, and this forms the basis of the transformation assay *(Combes R. et al., 1999)*.

The protocol for the SHE cells has undergone several modifications since it was developed by Berwald and Sachs *(Berwald Y. and Sachs L., 1965)*, the most extensive of which concern the changes introduced by LeBoeuf *et al. (LeBoeuf R.A. et al., 1999)*. Early versions of the assay included the use of an S9 metabolising system for the identification of procarcinogens *(Pienta R.J., 1979)*. The standard, historical assay involves 7-days treatment of the cells at clonal density with test chemical in Dulbecco's modified Eagle's medium, at pH 7.1-7.3 and containing foetal bovine serum. Colonies are then fixed, stained and scored for morphological transformation.

The SHE cell assay has several characteristics, which have discouraged its use in routine carcinogen screening, including *(Combes R. et al., 1999)*: a) low frequencies of morphological transformation following exposure to carcinogens that needs the scoring of large numbers of colonies to allow statistical procedures to be applied; b) wide discrepancies in the ability of different cell isolates and serum lots to support chemically induced morphological transformation; c) difficulties in scoring and identifying colonies of transformed cells; d) a general lack of dose-response relationship for chemical-induced transformation, which in most cases is the result of low transformation frequency.

Several of the reported problematic characteristics have been reduced by modification of the assay such as the use of Dulbecco's modified Eagle's medium at pH = 6.7 to culture the SHE cells *(Kerckaert G.A. et al., 1996; LeBoeuf R.A. and Kerckaert G.A., 1987; LeBoeuf R.A. et al., 1996)*, which has been shown to result in a 5-10-fold increase in transformation frequencies, a decrease in susceptibility to fluctuations in serum quality, and less ambiguity in the scoring of the transformed phenotype. The mechanism of enhanced transformation of SHE cells at pH 6.7 is not fully understood but is closely associated with intracellular acidification. Several resultant cellular effects appear to be important. These include decreased gap junctional intercellular communication, intracellular H^+ acting as a mitogenic stimulus, the cells taking on a more fibroblast, spindle-shaped appearance at pH 6.7 compared to higher pH. This means the criss-cross pattern of growth of the morphological transformation phenotype easier to score and reducing scored variability. Finally, lower pH should help to maintain cells in a less differentiated state and hence more susceptible to transformation *(LeBoeuf R.A. et al., 1990)*.

1.4 Relative Performance of Balb/c3T3 and SHE Transformation Assays

The databases for SHE and Balb/c3T3 cell transformation assays are more extensive and recent than for C3H/10T½ cell transformation system. It has been noted that, despite problems with these assays, and the fact that the protocols used were not necessarily optimised, the overall ability to identify carcinogens is encouraging *(Combes R. et al., 1999)*.

At the moment, the results reveal an overall concordance of 83% and 71%, sensitivity of 83% and 80%, and specificity of 82% and 60% for SHE and Balb/c3T3 assays, respectively. This performance was greatly superior to the performance of the *Salmonella* assay with the same set of chemicals *(LeBoeuf R.A. et al., 1999)*. Both assays detect mutagenic carcinogens with high sensitivity (94-95%), and overall, both have high negative predictivity in relation to the results of genetic toxicity assays, with 71% for the Balb/c3T3 assay and 79% for the SHE assay (Table 1.II). An analysis of data for 84 carcinogens and 77 non-carcinogens, showed for Balb/c3T3 an overall concordance of 71%, with a sensitivity of 80% and a specificity of 60% *(Matthews E.J., 1993c; Matthews E.J. et al., 1993a; Matthews E.J. et al., 1993b)*. A comparison with the corresponding *Salmonella* data reveals that both the SHE and the Balb/c3T3 assays can detect non-mutagenic carcinogens, with the SHE assay having a higher sensitivity for this class of chemical and a higher specificity for mutagenic non-carcinogens.

However, caution must be exercised in direct comparisons of the methods, as not all of the chemicals tested in the two systems were the same and thus, the composition of the set of chemicals being evaluated can greatly influence their performance. Furthermore, the two assays measure different stages in transformation, the clonal SHE assay assessing changes that occur early in the process and the Balb/c3T3 assay detecting the final stage in transformation, like the conversion of an immortal cell line to malignancy *(Kerckaert G.A. et al., 1996; Kerckaert G.A. et al., 1998; LeBoeuf R.A. et al., 1996; Matthews E.J. et al., 1993b)*.

Table 1.II: Evaluation of SHE and Balb/c3T3 assays [a]

Assay	Number of chemical tested	Overall concordance (%)	Sensitivity (%)	Specificity (%)	Positive predictivity (%)	Negative predictivity (%)
Overall results						
SHE (low pH)	75	83	83	82	89	79
Balb/c3T3	147	71	80	60	70	71
Chemicals mutagenic in *Salmonella*						
SHE (low pH)	27	93	95	88	95	88
Balb/c3T3	69	73	94	30	73	70
Chemicals non-mutagenic in *Salmonella*						
SHE (low pH)	48	77	75	80	84	70
Balb/c3T3	75	69	64	74	66	72

a: Kerckaert G.A. et al., 1996; Kerckaert G.A. et al., 1998; LeBoeuf R.A. et al., 1996; Matthews E.J. et al., 1993b.

By describing the relative performance of assays used for predicting carcinogenicity, it is unrealistic to expect that any one assay could be used to detect all mechanisms of cancer induction, due to the complexity of the cancer process. However, the high sensitivity of transformation assays demonstrated for a wide variety of chemical classes, presumably acting via a variety of mechanisms, indicates that a large proportion of chemical carcinogens act at the cellular level and can be detected *in vitro (LeBoeuf R.A. et al., 1999)*.

1.5 Human Cell-based Transformation Systems

Although the mechanisms responsible for rodent and human carcinogenicity are not fully understood, it is suggested that the sequence of processes for the transformation of rodent and human cells is similar, or nearly identical *(Fusenig N.E. and Boukamp P., 1994)*. However, there are species differences between rodents and humans, which could be important with regard to cancer development. These include: a) the fact that human is a long-lived species with cancer arising with increasing frequency

after 35 years of age, whereas rodents live for only 2-3 years; b) different metabolism of xenobiotics; c) much higher frequencies of spontaneous and induced immortalisation and transformation of rodent cells in culture, compared to human cells.

In order to incorporate the data in the risk assessment procedure an ideal transformation assay would utilise human cells. So far, it has proved to be extremely difficult to develop human cell transformation systems with tumorigenicity as the ultimate endpoint. The main problem seems to be that, unlike animal cells in culture, cultured human cells do not spontaneously give rise to immortalised cells. Since the carcinogenic process involves the sequential selection of cells with appropriate mutations in oncogenes and/or tumour suppressor genes, non-immortalised cells senescence before they have acquired all the genetic changes necessary for tumorigenicity. Thus, the currently available human cell transformation systems involve the use of genetically altered cell lines, rather than primary cultures, in which the cells have acquired an immortalised phenotype *(Fusenig N.E. and Boukamp P., 1994; McCormick J.J. and Maher V.M., 1989)*.

1.6 HaCaT Cell Line

The HaCaT cells are a human cell transformation system developed and studied together with the MSU-1 human fibroblast cell transformation model *(McCormick J.J. and Maher V.M., 1994; O'Reilly S. et al., 1998)*. The HaCaT cell line was derived by spontaneously immortalisation of normal human keratinocytes, most probably due to mutations in the p53 gene, and the consequent loss of senescence genes *(Boukamp P. et al., 1988; Boukamp P. et al., 1997)*. The name, HaCaT, indicates that it had developed from human adult skin keratinocytes during prolonged cultivation at a reduced Ca^{2+} concentration and elevated temperature. Unfortunately, there are no defined *in vitro* criteria for distinguishing between tumorigenic and non-tumorigenic HaCaT cells. Nevertheless, this immortalised cell line is a convenient model for studying tumour progression by various carcinogenic agents *(Fusenig N.E. and Boukamp P., 1998)*.

This spontaneously immortalised cell line (>140 passages) maintains *in vitro* a stable non-tumorigenic phenotype. However, the HaCaT cells can be transformed to tumorigenic variants, which exhibit both benign and malignant phenotypes by *ras*

oncogene activation and by several other interventions *(Boukamp P. et al., 1995; Fusenig N.E. and Boukamp P., 1998)*.

Both at genetic and phenotypic level, this model fits very well the known stages of human epithelial skin carcinomas *in situ* as well as of derived carcinoma cell lines. This allows genetic alterations and phenotypic characterisations associated with different stages of transformation to be identified.

2 *Apoptosis and Necrosis*

Programmed cell death or apoptosis is a process in which cell death is initiated and completed in an orderly fashion through the activation of various apoptotic pathways. During the last decade, there has been overwhelming interest in apoptosis and elucidation of mechanisms controlling this process. Apoptosis is an essential process required for development, morphogenesis, immune regulation, tissue remodelling, and some pathological reactions. Most apoptotic cells are characterised by unique biochemical and morphological features: cell shrinkage, phospatidylserine externalisation on the plasma membrane, release of cytochrome c from mitochondria, caspase activation, nuclear and cytoplasm condensation, plasma membrane blebbing, DNA fragmentation, and formation of apoptotic bodies that can taken up and degraded by neighbouring cells *(Allen R.T. et al., 1997)*. Apoptosis-associated nuclear condensation is usually accompanied by the activation of nucleases that first degrade chromosomal DNA into large 50 to 300 kb subunits and then into smaller units of ~180 bp *(Wyllie A.H., 1980)*. Because plasma membrane integrity is maintained during apoptosis, which prevents the leakage of cytosolic contents into the extracellular domain, this form of cell death is normally not associated with an inflammatory response. In contrast to apoptosis, necrosis is a passive form of cell death associated with inflammation, often resulting from an overwhelming cellular insult that causes cell and organelle swelling, breackdown of the plasma membrane, release of lysosomal enzymes, and spillage of the cell contents into the extracellular milieu *(Trump B.F. and Berezesky I.K., 1995)*. While apoptosis and necrosis can occur simultaneously, studies suggest that intracellular energy levels are important determinants of the mode of cell

death *(Leist M. et al., 1997)*.

There are two key elements in the apoptotic cascade located in the cytoplasm: the mitochondrion and the caspases, a family of cysteine proteases. Depending on the use of different initiating caspases, signal-induced apoptosis can be roughly divided in two categories: receptor-mediated 'extrinsic' apoptosis and mitochondria-mediated 'intrinsic' apoptosis. Whereas procaspase-8 is activated by receptors for Fas ligand and TNF (Tumour Necrosis Factor) through the recruitment of intracellular death domain-containing proteins, procaspase-9 is initiated by cytochrome c released from damaged mitochondria (Figure 2.1). Both activated caspase-8 and caspase-9 use the same executive caspases, mainly caspase-3, to complete an apoptotic process. All caspases are expressed as inactive precursors and are activated by cleavage at specific peptide bonds. There are a number of cellular proteins, which act as roadblocks in the activation cascade of caspases. These proteins act by counteracting the effect of caspases (such as cIAP1, cIAP2 and XIAP) or stabilising the outer membrane of mitochondria (such as Bcl-x and Bcl-2) *(Chen F. et al., 2001)*.

The apoptosis mediated by mitochondria may be more important and relevant in metal-induced cell death *(Ashkenazi A. and Dixit V.M., 1998; Green D.R. and Reed J.C., 1998)*.

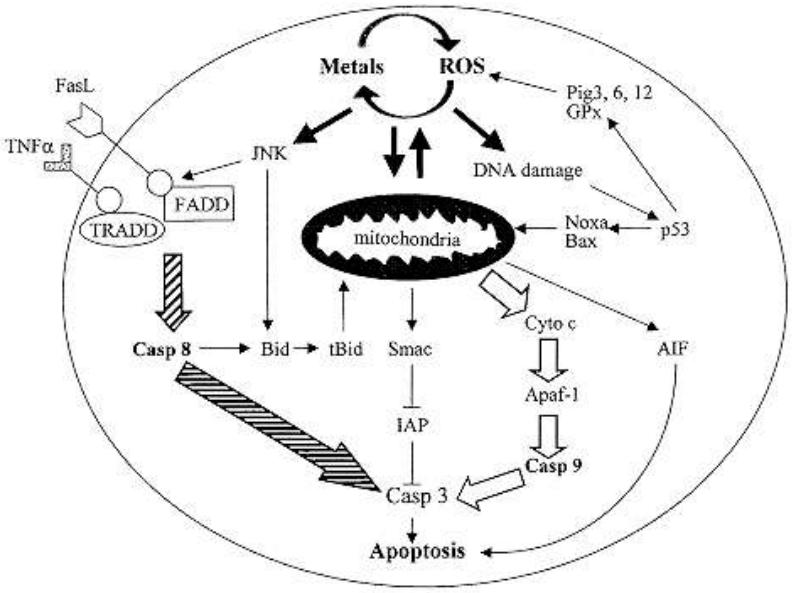

Figure 2.1: Apoptosis signalling pathway mediated by metals

(Chen F. et al., 2001)

2.1 Metals and Apoptosis

It is known that metals under certain circumstances are apoptotic. Current knowledge with respect to the apoptotic actions of As-, Cr- and Pt-compounds has been drawn from experimental, epidemiological and clinical studies *(Chen F. and Shi X., 2002)*.

Arsenic. The actions of arsenic are likely to be related to cell type arsenic species, length and dose of exposure. Inorganic As(III) is a well-documented carcinogen that also appears to be a valuable therapeutic tool in cancer treatment *(Bode A.M. and Dong Z., 2002)*. In fact, the apoptotic effect of As(III) was originally observed in human acute promyelocytic leukaemia cells *(Chen G.Q. et al., 1996)*. Therefore, arsenic induces apoptosis and may specifically target tumour cells. In addition, it influences distinct signalling pathways involved in mediating proliferating or apoptosis, including mitogen-activated protein kinases (MAPK) *(Bode A.M. and Dong Z., 2000)*, activator protein-1 or nuclear factor kappa B (NF-κB) *(Bode A.M. and Dong Z., 2002)*. Although the mechanism remains unclear, it has been consistently shown that p53 activation may not be involved in As(III)-induced cell apoptosis under many circumstances *(Chen G.Q. et al., 1996)*. Studies have suggested an oxidative stress model for As(III)-induced apoptosis *(Chen Y.C. et al., 1998)*. Moreover, arsenic-induced apoptosis is commonly associated with depletion of cellular GSH *(Bode A.M. and Dong Z., 2002)*, decreased mitochondrial membrane potential accompanied by cytochrome c release *(Cai X. et al., 2000)*, caspase activation *(Chen Y.C. et al., 1998)*, changes in Bcl and Bax proteins and DNA fragmentation induction *(Larochette N. et al., 1999)*.

Chromium. In cultured mammalian cells Cr(VI)-compounds are indicated as potent inducers of apoptosis. Both sodium chromate and lead chromate increase the translocation of phosphatidylserine in a dose-dependent manner *(Martin S.J. et al., 1995)*. Electron microscopic analysis of Cr-treated cells has revealed classical hallmarks of apoptosis, such as chromatin condensation and margination, membrane blebbing, extensive vacuolisation (indicative of cell stress), apoptotic bodies that are remnants of apoptotic cells and uptake of apoptotic cells by surrounding normal cells *(Singh J. et al., 1998)*. Then, studies on CHO cells report sodium chromate as inductor of internucleosomal fragmentation of cellular DNA in a dose-dependent fashion *(Manning F.C.R. et al., 1994)*. The mechanisms by which chromium induces apoptosis are not

fully understood. Most of the effects of chromium at the cellular level (e.g. oxidative stress) as well as the molecular level (e.g. DDC, transcriptional inhibition) have the potential of triggering apoptosis *(Singh J. et al., 1998)*. Evidence indicates that depending on the rate, magnitude and spectrum of genotoxicity and mitochondrial damage, cells exposed to Cr(VI) are fated to undergo either terminal growth arrest or p53-dependent apoptosis, which is considered to be one of the critical steps in the induction of apoptosis by Cr(VI), although p53-independent apoptosis induced by Cr(VI) has also been reported *(Carlisle D.L. et al., 2000)*. Further experiments were performed using specific peptide inhibitors to caspases-1 and –3. It was observed that caspase-3, but not caspase-1, may play a role in Cr-induced apoptosis *(Singh J. et al., 1998)*.

Pt-compounds. Apoptosis of individual cells may represent a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells or excess that have improperly been induced to divide by a mitotic stimulus. This notion is supposed by the fact that both established carcinogens and commonly used chemotherapeutic drugs can induce this form of cell death *(Hickman J.A., 1992)*. Cisplatin and carboplatin are among the most active and widely used cytotoxic anticancer drugs. Apoptosis is observed as a pattern of cell death caused by cisplatin. The cellular factors that render cells more sensitive (or resistant) to cisplatitn should include p53 status and nucleotide excision repair machinery *(Coleman A.B. et al., 2000)*. Morphological changes characterised by condensation and fragmentation of chromatin, DNA cleavage by oligonucleosome-size DNA fragments and expression of caspase-3 activity *(Sekiguchi I. et al., 1996)* are demonstrated for cisplatin. Interestingly, recent experiments indicate that carboplatin induces less apoptosis than cisplatin. In fact, carboplatin is a successful drug in anticancer therapy although used at concentrations up to five times higher than those for cisplatin *(Watanabe K. et al., 2002)*.

3 Trace Metals and Carcinogenesis

Essentiality of trace metals. Trace metals play an important role for human and animal life. Some elements can be highly toxic to various life forms; others are considered essential, but can become toxic at higher doses. The biochemical effects of trace metals *in vivo* are specific. A deficiency of one element can be prevented and remedied only by that, but not by another element, even if the latter is chemically related to the first. Certain trace metals in humans are essential for growth and reproducibility of the organism by: a) carrying ion messages (Na, K, Ca); b) triggering proteins (Ca, Mg); c) electron transport (Mn, Fe, Cu); d) catalysis (Cr, Fe, Cu, Zn, Mo). The function of metals in a biological system can only be appreciated in terms of the compartments into which these elements are placed by the use of metabolic energy. Table 3.I shows the concentration of elements in different compartments *(Williams R.J.P., 1989*).

Table 3.I: Distribution of metals

(Williams R.J.P., 1989)

Extracellular	Intracellular	Cytoplasmic
Na, Ca	K, Mg	K, Mg
Cu, Mo	Fe, Co	Co
Al	Zn, Ni, Mn, Se	Zn, Se

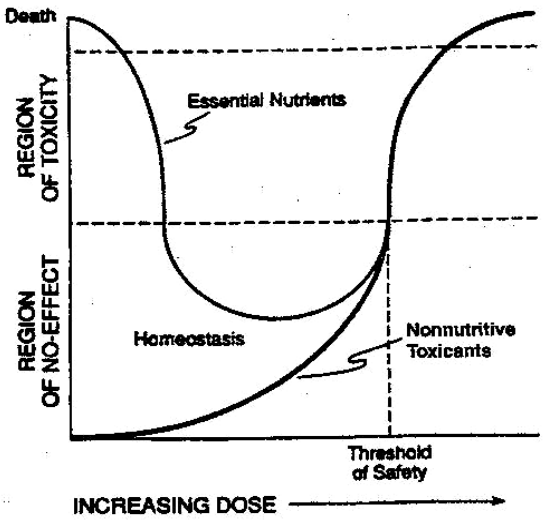

Figure 3.1: Dose-response curve for trace metals

(Eaton D.L. and Klaassen C.D., 1996)

The typical U-shape dose-response curve for an individual exposed to trace metals is shown in Figure 3.1 Dose-exposure less than that required for normal physiological function leads to adverse effects. This region of the dose-response relationship for essential nutrients is commonly referred as "deficiency". As the dose increases to a point where the deficiency no longer exists, no adverse response is detected and the organism is in a state of homeostasis. However, as the dose is increased to abnormally high levels, an adverse response appears and increases in magnitude with increasing dose, just as with

other toxic substances *(Eaton D.L. and Klaassen C.D., 1996)*.

Toxicity and carcinogenicity of trace metals. Trace metals can exert their toxic action by several biochemical mechanisms as listed below:

Mechanisms of toxicity:

- One metal may substitute for another, essential metal, e.g. in an enzyme, resulting in toxic effects.
- Most metals have high affinity for a wide variety of organic ligands to form complexes with high stability constants. This can change the conformation of enzymes and decrease the activity.
- Certain metals can act as catalysts, not as part of an enzyme but in their own right.
- Oxyanions of metals (e.g. CrO_4^{2-}) are coordinate complexes with a specific stereochemical configuration leading to interactions with the molecular target or transport proteins (e.g. for PO_4^{3-}).
- Certain metals form organometallic compounds with at least one covalent stable bond with the carbon atom. They can be ruptured only by special enzymes.
- The action of uranium on the proximal tubular cells of kidney nephron is an example of toxicity caused by selective accumulation of a metal.

Factors influencing toxicity:

- Different compounds ("species") of the same metal ion may essentially differ in toxicokinetics and toxicodynamics.
- Various oxidation states of a metal.
- Extent of absorption, e.g. from the gastrointestinal or respiratory tract.
- Particle size of the metal or its compound (especially important for inhalation toxicity).
- Distribution via the blood to the various organs.
- Extent and the route of excretion, as well as the influence of metabolism and detoxification processes.
- Storage in the cells in the form of harmless or harmful particles.
- Concentration of the metal in organs.
- pH of body fluids and organs.
- Ability of other metals or body compounds to increase or reduce metal toxicity.
- Age of the individual.
- The individual's general health.
- Inherited or acquired metabolic disturbances.

Trace metals can also induce cancer. From a genetic point of view, the most serious reaction of metals with DNA is the induction of crosslinks between both DNA strands. Chelation or formation of complexes between DNA and metals at nucleophilic DNA sites and binding to the phosphate groups could also be detected. The reactions of

metal ions with amino and sulphydryl groups of proteins involved in nucleic acid metabolism can affect replication fidelity and thus, indirectly alterations in genetic information. Metal compounds can also induce DNA strand breaks by causing depurination of DNA or via their involvement in generating oxygen free radicals *(Fraústo da Silva J.J.R. and Williams R.J.P., 1991)*. All these mechanisms can be responsible for the carcinogenic action of metals. On the basis of epidemiological and experimental animal data, international agencies have proposed a classification of agents on the basis of the evidence for their carcinogenicity in humans. The first scheme that was generally recognised was devised by the International Agency for Research on Cancer (IARC) (Table 3.II), on the basis of the demonstration of the carcinogenicity of an agent *(Pitot H.C. and Dragan Y.P., 1996)*.

***Table 3.II: IARC classification of carcinogenicity of metals and metal compounds in humans** (Pitot H.C., 1986)*

Group	Evidence	Metals / Metal compounds
1. Carcinogenic	Sufficient (human)	Aluminium, arsenic, beryllium, cadmium, chromium, iron, nickel compounds
2A. Probably carcinogenic	Limited (human) Sufficient (animal)	Cisplatin
2B. Possibly carcinogenic	Limited (human) Inadequate (human) Sufficient (animal)	Antimony trioxide, cobalt, lead, methylmercury compounds, metallic nickel
3. Not classifiable as carcinogenic		Manganese, antimony trisulfoxide, organolead compounds, metallic mercury and inorganic mercury compounds, selenium, titanium dioxide
4. Probably not carcinogenic	Inadequate (human) Inadequate (animal)	

The terminology that is utilised means *(Pitot H.C., 1986)*:

a) *Sufficient evidence* of carcinogenicity: there is a casual relationship between the agent or agents and human cancer.

b) *Limited evidence* of carcinogenicity: a casual interpretation is credible even though alternative explanations such as chance, bias, and confounding variables could not be completely excluded.

c) *Inadequate evidence* of carcinogenicity: one of the following conditions prevailed: 1) there were few pertinent data; 2) the available studies, while showing evidence of association, did not exclude chance, bias, or confounding variables; 3) studies were available that did not show evidence of carcinogenicity.

Other classification schemes exist including those of the U.S. Environmental Protection Agency (EPA), the Chemical Manufacters Association, and the European Community (EC). These classifications agree with respect to the classification of compounds that are known human carcinogens but place different emphases on the results of animal and genotoxicity studies.

In this context, specific examples of trace metals showing carcinogenic potential are arsenic, chromium and some Pt-compounds. On the basis of the IARC classification, arsenic and chromium belong to group 1, whereas cisplatin to group 2A *(IARC, 2001)*. Hereafter, few essentials concerning information on toxicity and carcinogenicity of these metals, investigated in the present study, are reported.

Arsenic. Arsenic (As) is often viewed as synonymous with "toxic". The evil reputation is probably undeserved considering how well arsenic has served mankind. Salvarsan (an organoarsenic compound) was a highly renowned chemotherapeutic agent against syphilis and other venereal diseases, and for many years, Fowler's solution (1% solution of arsenic trioxide) remained one of the most dispensed medicaments in the Western culture. Adverse health effects caused by arsenic compounds have long been recognised, including neurotoxicity, liver injury, peripheral vascular disease (known as Blackfoot disease), and increased risk of cancer *(Chen C.J. et al., 1995)*. Significant exposures to arsenic occur in a variety of workplaces such as lead smelters, glass works, and use of arsenic-containing agricultural products such as pesticides and herbicides. Inhalation and ingestion of As-compounds have been primarily associated with increased incidences of a various cancers such as skin, lung, bladder, and liver cancer

(Chiou H.Y. et al., 1995).

One of the main sources of exposure to arsenic, mostly to inorganic As-compounds, is contaminated drinking water. This problem affects mainly the populations of Asia (Bangladesh, China, India, Thailand, Taiwan) *(Chen C.J. et al., 1999)* and South America (Chile, Argentina) *(Smith A.H. et al., 2000)*. The concentration of inorganic arsenic in drinking water in these regions of the world dramatically exceeds the standard (50 μg/l) adopted in 1987 by the US Environmental Protection Agency (USEPA), and the number of the exposed comes to millions of people *(Gradecka D. et al., 2001)*. It is striking that the different populations exposed to the similar inorganic arsenic levels in drinking water vary in individual susceptibility. Moreover, the results of other studies show that some populations may be insensitive to carcinogenic effects of inorganic arsenic, and in spite of high exposure, the increased cancer incidence has not been observed *(Aposhian H.V. et al., 1997)*. On the other hand, it has been demonstrated that over a one-thousand-year exposure of many generations of natives from Northern Chile (Atacameno people) to inorganic arsenic does not protect them from malignant diseases *(Smith A.H. et al., 2000)*. The various individual responses in populations may result from the presence of other factors promoting the carcinogenic process. They may include dietary, environmental (UV radiation), and genetic factors (polymorphism of certain enzymes, primarily methyltransferases) *(Vahter M., 1999)*. Identification and understanding of the mechanism of the toxic effect of inorganic As-compounds may be helpful in estimating cancer risk and preventing malignant and other diseases. There are many hypotheses on this issue that are not yet fully clarified and still arise a lot of controversy among researchers. It is believed that inorganic As-compounds do not affect DNA directly; they form adducts with DNA or induce DNA-protein cross-links. Exposure to arsenic *per se* does not cause point mutations, which are observed during simultaneous exposure to arsenic and physical factors (UV radiation, X-radiation or gamma radiation), as well as to some chemical compounds (n-methyl-n-nytrosourea). This means that inorganic arsenic is a co-mutagen and enhances mutagenic process of other agents. Some authors believe that the toxicity of inorganic As-compounds results from the inhibition of the activity of DNA repair enzymes. This is caused mainly by physical and chemical properties of arsenic and its ability to bind sulfhydryl groups present in the enzymes *(Gradecka D. et al., 2001)*. However, arsenic has not been shown conclusively to be an initiating or a

promoting agent of carcinogenesis in animals and in contrast to classic tumour promoting agents its effects are not reversible.

Comparison of chromosome aberration frequencies induced by trivalent and pentavalent arsenic have indicated that the trivalent forms are far more potent and genotoxic than the pentavalent forms *(Barrett J.C. et al., 1989)*. The other data also suggest that inorganic As-compounds are responsible for altered gene expression, which is caused by the induction of oxidative stress and/or disturbance of DNA methylation. The increased expression of genes responsible for positive regulation of cell cycle and simultaneous inhibition of the expression of negative regulation genes was also observed during excessive cell proliferation caused by exposure to inorganic arsenic. These genetic abnormalities usually lead to the development of malignancies. Thus inorganic arsenic is carcinogenic, although it appears to be extremely effective as a chemotherapeutic agent in treating certain types of cancers. In particular, arsenic has been very useful in treating relapsed or all-*trans*-retinoic acid (ATRA)-resistant acute promyelocytic leukemia (APL) patients *(Bode A.M. and Dong Z., 2002; Gradecka D. et al., 2001)*. Moreover, arsenic induces cell death and may specifically induce apoptosis in certain types of tumour cells *(Bode A.M. and Dong Z., 2002)* (Section 2.1).

Toxicity and carcinogenicity of arsenic are strongly depending on its chemical form. Arsenic may exist in three different oxidation states, namely, as the metalloid (0 oxidation state), as arsenite (trivalent or +3 oxidation state), and as arsenate (pentavalent or +5 oxidation state). Different arsenic-containing compounds vary substantially in their toxicity to mammals. Arsine gas (or arsenous hydride, AsH_3) is clearly the most toxic, followed in order of generally decreasing toxicity by inorganic trivalent compounds → organic trivalent compounds or arsenoxides → inorganic pentavalent compounds → organic pentavalent compounds or arsonium compounds → elemental arsenic. Some arsenic compounds are apparently not toxic at any dose *(Gorby M.S., 1994)*.

Since inorganic trivalent species are toxic and carcinogenic to humans, the body has developed detoxication mechanisms for such As-species. Although trivalent arsenic can be oxidised to the less toxic pentavalent form in natural system, the usual bodily biotransformation leads in the other direction, to the formation of trivalent arsenic *(Morton W.E. and Dunnette D.A., 1994)*. These changes include reduction of As(V) to As(III) and methylation of these forms to pentavalent/trivalent monomethylarsonic

(MMA) and dimethylarsinic (DMA) acid *(Vahter M., 1999)*:

$H_2As^{V}O_4^{-} \Leftrightarrow H_3As^{III}O_3 \Rightarrow \mathbf{CH_3As^{V}O_3^{2-}}$ **(MMA)** $\Leftrightarrow CH_3As^{III}O_2^{2-} \Rightarrow \mathbf{(CH_3)_2As^{V}O_2^{-}}$ **(DMA)** $\Rightarrow$

Methylation is regarded as a detoxification process. However, the discovery of toxic trivalent methylated forms in methylation pathways raises the question if biomethylation is really a detoxification process *(Aposhian H.V., 1997)*.

The paucity of information regarding the mechanism of carcinogenic action of arsenic has been attributed to the apparent lack of valid or appropriate experimental animal models. Thus, much of the current knowledge pertaining to the actions of arsenic has been drawn from epidemiological and clinical studies. In addition, to being cell and tissue type specific, research data indicate fairly conclusively that the actions of arsenic are related to several other variables including arsenic species and length and dose of exposure *(Bode A.M. and Dong Z., 2002)*.

Chromium. Chromium (Cr) is found in group VI A of the periodic scale and is a member of the first transition series. Its valences range from 2- to 6+. In acid solution Cr^{2+} (chromous), Cr^{3+} (chromic), and $Cr_2O_7^{-2}$ (dichromate) are representative, while in basic solution $Cr(OH)_2$, CrO_2^{-1} (chromite), and CrO_4^{-2} (chromate) are representative. The chromous ion is rapidly oxidised to Cr(III) by air and slowly by H^+ *(Sanderson C.J., 1982)*.

The most common oxidation states of chromium are 3+ and 6+. However, organic chromium is considered to be the biologically active form of chromium. Cr(III) combined with nicotinic acid and other amino acids is speculated to be the glucose tolerance factor (GTF) that acts as a potentiating agent for insulin. In fact, the best-known biological function of chromium, the maintenance of normal glucose tolerance, has been described and the existence of chromium deficiency in several populations groups, particularly in elderly people and in malnourished children has been established. Therefore, increasing emphasis is being placed on the potential protective role of chromium in preventing the development of impaired glucose tolerance, diabetes mellitus and cardiovascular disease *(Guthrie B.E., 1982)*.

Some of the important factors in determining the biological outcome of chromium exposure include the bioavailability, chemical speciation and solubility of

Cr-compounds, intracellular reduction, and interaction of chromium with DNA. In this context, exposure to environmental chromium is an emerging concern. Some Cr-compounds are documented human carcinogens *(IARC, 1990)*. The greatest exposures to carcinogenic chromium occur in an occupational setting, and individuals in the chromate and dichromate industries have 29-fold overall increase in respiratory cancer (e.g. lung cancer) *(Blankenship L.J. et al., 1997)*.

Chromium exposure is also associated with respiratory toxicity manifesting as respiratory distress, perforation of the nasal septum and respiratory tract and skin ulcerations. With the prolonged exposures that may occur in an occupational or environmental setting, chromium may accumulate in different cellular compartments such as nucleus and mitochondria and cause several effects described ahead *(Ono H. et al., 1981; Ryberg D. and Alexander J., 1990)*. Thus, occupational and environmental exposure to chromium by inhalation is associated with long-term carcinogenic conseguences as well as short-term toxic manifestations in the lungs. Moreover, the carcinogenicity of specific Cr-compounds is influenced by both the valence and the solubility of the chromium species. Earlier studies indicated that the slightly soluble to highly insoluble particulate chromates of lead, zinc and calcium were capable of inducing tumours in rat lungs, while soluble chromates such as potassium dichromate were the least carcinogenic *(Singh J. et al., 1998)*.

The stable oxidation states of chromium found in nature are Cr(III) and Cr(VI). Cr(III) is unable to enter cells but Cr(VI) enters into cells through membrane anionic transporters. Intracellular Cr(VI) is metabolically reduced to the ultimate Cr(III). In cellular systems, Cr(VI) is reduced by certain flavoenzymes (glutathione reductase) to generate Cr(V). During this process, molecular oxygen is reduced to $O_2^{\cdot-}$, which generates H_2O_2 via dismutation. The resultant Cr(V) reacts with H_2O_2 to generate $^{\cdot}OH$ radical via a Fenton-like reaction *(Ye J. et al., 1999)*. The reduction products (e.g. ROS, the intermediate oxidation states Cr(V) and Cr(IV) as well as the ultimate reduced form, Cr(III)) elicit a broad spectrum of genotoxic effects. Forms of chromium-induced structural damage of genomic DNA include: a) 8-oxodeoxyguanosine, a form of oxidative DNA damage; b) DNA-DNA interstrand cross-links (DDC); c) Cr-DNA adducts; d) single-strand breaks; e) DNA-protein cross-links (DPC); f) chromosomal aberrations. These DNA lesions result in various types of functional damage such as: a) DNA polymerase arrest and inhibition of DNA replication; b) RNA polymerase arrest

and transcriptional inhibition; and c) inhibition of DNA topoisomerases *(Singh J. et al., 1998)*. This leads to diverse cellular effects such as mutagenesis, cell cycle arrest, apoptosis and neoplastic transformation (Figure 3.2). In particular, it has been suggested that apoptosis needs to be considered a component of chromium induced multistage carcinogenesis (Section 2.1). This means that the mode of cell killing by this agent is important because treatments that reduce cell survival must be utilised experimentally in order to detect the cellular mutagenicity and carcinogenicity of this compound *(Blankenship L.J. et al., 1994)*.

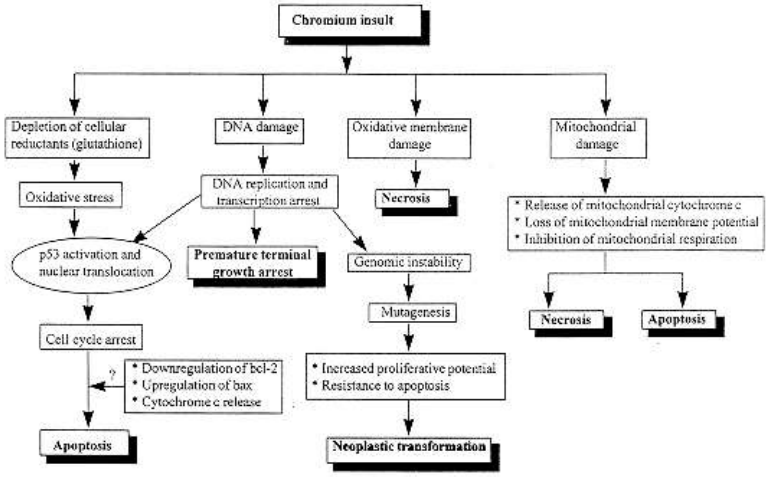

Figure 3.2: Cellular response to chromium

(Singh J. et al., 1998)

Platinum Group Metals. The elements ruthenium, rhodium, palladium, osmium, iridium, and platinum form the so-called platinum group metals (PGM) of the periodic table.

Environmental, occupational and biomedical exposure of Pt-compounds. Pure metal forms of platinum (Pt), palladium (Pd) and rhodium (Rh) and their alloys are used as corrosion-resistant materials for melting tubes, laboratory instruments, and spinning jets in synthetic fiber production. Other applications include uses as catalysts in chemical synthesis, in car mufflers, electrochemical industry, petrol, jewellery, and they also find use as alloys in dentistry and orthopaedics *(König K.H. and Schuster M., 1994)*. The platinum content in the atmosphere has been monitored in platinum mines, platinum refineries, at catalyst manufactures and, in general, the work environment. In badly ventilated work places, the platinum concentration can reach the $mg \cdot m^{-3}$ level, although concentrations at the $\mu g \cdot m^{-3}$ level or below are quite common *(Shi Z.C., 1998)*. The analysis of Pt, Pd and Rh in urban airborne particulate matter is challenging, since these new pollutants are expected to increase in the environment in proportion to the

Figure 3.3: "Three way" catalytic converter

increase in the number of cars fitted with catalytic converters. These elements partially leave the surface of the catalyst during its life as a result of poorly known processes, including thermal and mechanical abrasion of the catalyst and are spread and bioaccumulate in the environment *(Gómez B. et al., 2001)*. This new technology based on a "three way" catalytic converters oxidises CO, unburned hydrocarbons and nitrogen oxides *(Pietra R. et al., 1994)* (Figure 3.3). This property together with the use of poor mixtures and the optimisation of the combustion cycle greatly reduces the automotive emission of pollutants. Platinum has been the most widely studied of the Pt-group elements. Relevant factors in the toxicity of Pt-compounds are their physicochemical properties, dose and route of administration *(Gómez B. et al., 2001)*.

Platinum toxicity is depending on the valence of the compounds and the electron structure. In metallic state platinum is non-toxic and non-allergenic, while in soluble divalent or tetravalent forms induces occupational diseases, such as asthma and contact dermatitis. However, interrupting the occupational contact with these salts considerably reduces the intensity of the clinical symptoms of the diseases while resuming the contact induces new exacerbation *(Roshchin A.V. et al., 1984)*. Moreover, the frequency of occurrence of skin affections has been found to depend on the concentration of the solutions of the metal compounds and on the concentration of dust of these compounds in the air of the working premises. The degree of this dependence increases with increasing concentration.

Platinum can act as an apten by binding high molecular weight carriers such as blood proteins that stimulate lymphocytes to produce specific antibodies against the allergen. Studies have shown Pt(II) as reactive with human transferrin and with sulphur atoms of albumine, the most abundant plasmatic protein. IgE antibodies are responsible for immediate effects of a second exposure, whereas IgG antibodies for the delayed effects *(Trynda L. and Kuduk-Jaworska J., 1994)*.

Therapeutic use of Pt-compounds. Although most modern pharmaceuticals are purely organic compounds, the use of metal-containing agents for both therapy and diagnosis is of increasing interest and relevance. In this context, since Rosenberg

published the first work on the antitumour activity of Pt-compounds 40 years ago, Pt-based drugs have made a major contribution to human cancer therapy *(Rosenberg B. et al., 1965)*. Actually, treatment of malignancies with the Pt-group of drugs is included in many chemotherapy regimens as part of standard treatment. There are a number of drugs within the Pt-group but clinically only two have so far been approved for treating malignant disease, mainly cisplatin (*cis*-diamineplatinum(II)dichloride) and carboplatin (*cis*-diamine-1,1-cyclobutane dicarboxylate platinum(II)).

They are classified as cell cycle non-specific chemotherapy agents, because their action is independent of the position of the cell in cell cycle. Cisplatin is a Pt(II) analogue. This molecule carries a pair of chlorine atoms, which react with various components of DNA, in particular with the nitrogen of guanine. The spatial arrangement of the platinum complex enables it to react with two different guanine moieties, thereby forming cross linkages (Figure 3.4). It tends to form linkages between guanine in the same strand and also with the opposite strand frequently referred to as inter-strand and intra-strand cross linkages. These would bind DNA strands together and prevent them from separating at the time of DNA replication. For both cisplatin and carboplatin it is envisaged that prior to exerting biological activity, the complex must be converted to reactive Pt-species by loss of one or both leaving groups, chloride and cyclobutane dicarboxylato, respectively. The aqua and hydroxyl species thereby formed react avidly with nucleophilic sites in macromolecules *(Selvaratnam G. and Philips R.H., 1997)*.

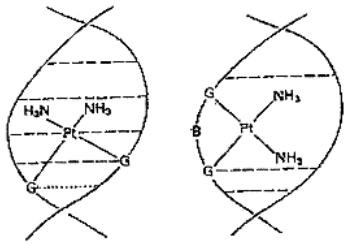

Figure 3.4: Inter- and intra-strand cross linkages of Pt(II) analogues

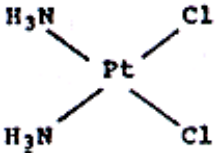

Figure 3.5: Cisplatin

Cisplatin (Figure 3.5) is active against testicular and ovarian cancer and it is also being used with some success in lung, bladder, cervix, and head and neck tumours. Unfortunately, during early clinical trials it was clear that although cisplatin was an active antitumour agent, it was also extremely toxic. Kidney damage and vomiting are serious problems and in addition, hearing loss, peripheral neuropathy, and anaemia are frequently seen. An alternative approach has been to control the toxicity of cisplatin by design of a "second generation" drug, from which carboplatin (Figure 3.6) was selected for clinical evaluation. This shows an equal

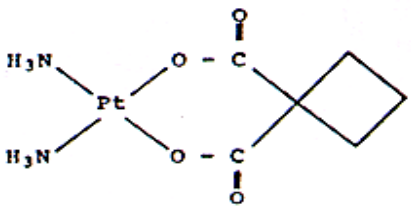

Figure 3.6: Carboplatin

or greater anti-tumour activity than cisplatin and it causes less nephrotoxicity, nausea and vomiting *(Selvaratnam G. and Philips R.H., 1997)*. Currently there are several platinum analogues in clinical trials around the world. These include ormaplatin, DWA2114R, enoplatin, lobaplatin, C1-973, 254-S, JM 216, and liposomal trapped platinum 1 L-NDDP *(Weiss R.B. and Christian M.C., 1993)*. At present, the development of a "third generation" analogue (such as ormaplatin and lobaplatin) has been designed to overcome cisplatin resistance, and in particular to produce oral biovailability. Recently, investigations have been carried out on the activity of Pt(IV)-compounds as anti-tumour agents to discover whether such compounds are real drugs or, as is widely believed, act as prodrugs, that is they are reduced to Pt(II) before reaching their DNA target *(Talman E.G. et al., 1998)*. Pt(IV) is kinetically more inert than Pt(II), which means Pt(IV) drugs are more stable to acidic media, so may survive the conditions present in the stomach, and thus can be administrated orally. Experiments to determine the *in vitro* reactivity of Pt(IV)-compounds detected identical Pt-DNA adducts generated from reaction with both Pt(IV) and Pt(II). This suggests that the mechanism of inhibition of DNA replication is the same for Pt(IV) and Pt(II). Reaction occurred in all cases, and Pt(IV)-DNA intermediates were found, so reduction to Pt(II) is not a requirement prior to reaction with DNA. They speculate that Pt(IV) may enter the cell by a different mechanism to Pt(II) and so the possibility remains that some Pt(IV)-compounds do not act as prodrugs *(Talman E.G. et al., 1998)*.

3.1 Hard Metals

The term "hard metals" indicates metal alloys mainly constituted of tungsten carbide (70% - 95%) with different percentages of other metal carbide (titanium, tantalum, chromium, vanadium) with cobalt as binder (5% - 25%). The composition of these alloys can vary depending on their particular use mostly in engineering, car and military industry.

Occupational exposure to hard metals dust induces asthma, lung fibrosis, and

produce a particular pathology called "Hard Metals Disease", which is not correlated with age, working time or exposure dose *(Sabbioni, E. et al., 1994)*. In this disease the immune reaction is triggered by cobalt that acts as an apten and its sensitisation in pulmonary fluid is increased by tungsten. Other synergistic or antagonistic effects due to the presence of the other constituent metals are unknown.

Therefore, exposure to hard metals represents a good example of multiple exposures (combined metal mixtures), whose cytotoxicity mechanisms are suitable to be studied *in vitro* by cell cultures.

3.2 Metal Speciation

Essential/toxic effects of trace metals depend strongly on the chemical form in which the element is present in the system. For example, Cr(VI) ions are considered far more toxic than Cr(III) *(Katz S.A. and Salem H., 1994)*, while organic (methylmercury) and inorganic mercury compounds show different patterns of toxicity. Often these different chemical forms of a particular element or its compounds are referred to as "species". Because numerous publications have appeared in which the term "speciation" is employed, we summarise here the mean of main terminology related to this important aspect:

a) *Chemical species. Chemical elements:* specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure.

b) *Speciation analysis. Analytical chemistry:* analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample.

c) *Speciation of an element; speciation.* Distribution of an element amongst defined chemical species in a system.

d) *Fractionation.* Process of classification of an analyte or a group of analytes from a certain sample according to physical (e.g. size, solubility) or chemical (e.g. bonding, reactivity) properties.

A recommendation is made to restrict the use of the term speciation to the distribution of an element among defined chemical species and to use the terms speciation analysis and fractionation to refer to analytical activities. When speciation

analysis is impractical, fractionation may still be useful *(Templeton D.M. et al., 2000)*.

Speciation strongly affects the determination of the impact that trace metals may have on human health that is a rather complicated task. One of the causes of this complexity is the ability of trace metals to exist in the natural environment in various chemical forms to which man can be exposed. In fact, some human activities release trace metals into the environment in physico-chemical forms different from those naturally present with the possibility of accumulation of their toxic species in food *(Bolm-Aurdoff U. et al., 1992)*. These compounds can interact with body cells leading to altered metabolic pathways and biochemical effects. Thus, toxicological research directed towards establishing dose-effect relationships for trace metals demands specific analytical procedures that can differentiate between their chemical and biochemical forms. Such a need to resolve the total concentration of trace metals into single chemical species poses great experimental difficulties and imposes the use of very sensitive analytical techniques for trace metals determinations after specific preparation procedures of the different chemical species *(Sabbioni E. et al., 1987)*.