17 Cytotoxicity Studies on HaCaT Cell Line

The present section reports the CFE experiments carried out on the HaCaT cell line, an *in vitro* model of immortalised human keratinocytes cells (Section 5).

$(NH_4)_2PtCl_6$, $(NH_4)_2PdCl_6$ and $(NH_4)_3RhCl_6$ (Section 17.1) as well as $NaAsO_2$, $Na_2HAsO_4 \cdot 7H_2O$ and $(CH_3)_3AsCH_2COO^-$ (Section 17.2) were tested for studying the corresponding dose-effect relationships.

17.1 Platinum, Palladium and Rhodium Compounds

Table 17.I shows the results obtained after 24- and 72-hour exposure of HaCaT cells to $(NH_4)_2PtCl_6$, $(NH_4)_2PdCl_6$, $(NH_4)_3RhCl_6$ (concentrations ranging from 1 μM to 100 μM).

Table 17.I: Cytotoxicity of Pt-, Pd- and Rh-compounds in HaCaT cells

Metal compound	Concentration (μM)	CFE	
		24 h	72 h
$(NH_4)_2PtCl_6$	Control	100	100
	1	79.6 ± 2.4	80.3 ± 2.3
	10	50.4 ± 3.9	70.0 ± 4.1
	100	0.7 ± 0.2	0
$(NH_4)_2PdCl_6$			
	1	101.0 ± 6.1	99.3 ± 3.8
	10	90.0 ± 3.7	96.5 ± 6.6
	100	85.3 ± 4.4	88.4 ± 5.1
$(NH_4)_3RhCl_6$			
	1	89.0 ± 5.5	95.5 ± 3.7
	10	71.4 ± 3.5	76.2 ± 2.7
	100	47.1 ± 2.3	45.7 ± 2.5

The stronger cytotoxicity of Pt(IV) in comparison to the effects of Pd(IV) and Rh(III) was confirmed at equimolar concentration of 10 µM both after 24- and 72-hour exposure. The IC_{50} value (after 72 h) for Pt(IV) was 30 µM, whereas the corresponding values for the other salts were 100 µM for Rh(III) and higher than 100 µM for Pd(IV).

17.2 Inorganic and Organoarsenic Compounds

A CFE experiment was carried out on the HaCaT cells in order to estimate the cytotoxic effect induced after 72-hour exposure to a wide range of concentrations of inorganic $NaAsO_2$ and $Na_2HAsO_4 \cdot 7H_2O$ as well as organic $(CH_3)_3AsCH_2COO^-$ arsenic species (Figure 17.1).

Figure 17.1: Cytotoxicity of inorganic and organoarsenic compounds in HaCaT cells

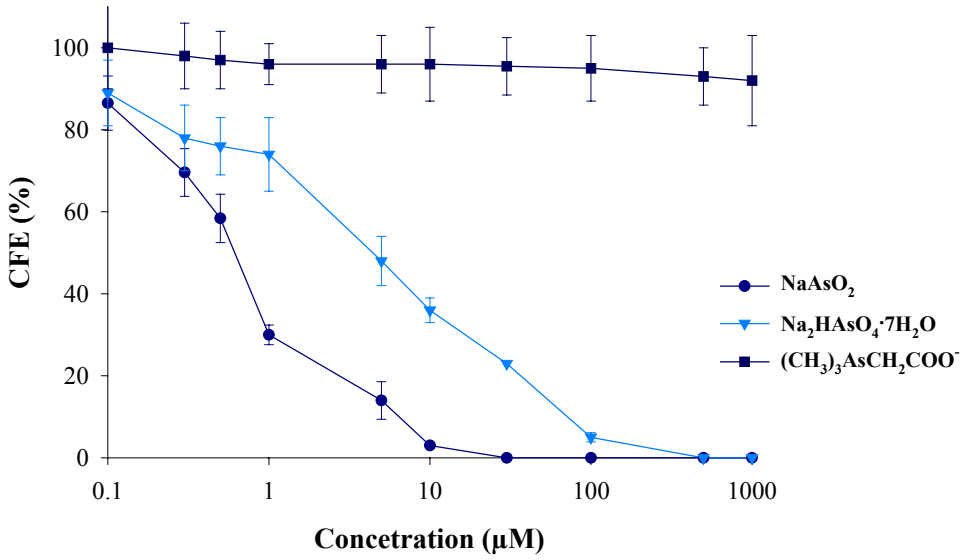


Figure 17.1 shows the dose-dependent inhibition of the colony formation in the HaCaT cells exposed to $NaAsO_2$ and $Na_2HAsO_4 \cdot 7H_2O$ and As-betaine for 72 hours.

As(III) was more toxic than As(V), showing IC_{50} values at 0.5 µM and 5 µM, respectively, while no significant cytotoxic effect was detected after exposure to As-betaine at the concentrations ranging from 0.1 µM to 1000 µM. Values of CFE obtained with this organic compound were always higher than 90% of controls.

18 Optimisation of Syrian Hamster Embryo Cell Line

The results described in the following section concern the primary rodent finite life span cell line (Syrian Hamster Embryo or SHE cells) (Section 6). The study was mainly focused on the optimisation and standardisation of the best culture conditions in order to assess the cytotoxicity of selected metal compounds for subsequent comparison with the Balb/3T3 cells. The optimisation study included the choice of the best combination of culture medium and serum (Section 18.1); the establishment of the pH value of culture medium (Section 18.2); the choice of the most suitable plastic type of culture dishes (Section 18.3).

18.1 Culture Medium and Serum

The best SHE culture maintenance was set up by checking combinations of MEM culture medium with foetal bovine serum of two different origins (Table 6.1). Over a week of culturing the medium was changed twice. The culture medium was added with 20% of serum and its initial pH value was pH = 7.4.

Table 18.I shows a negligible difference between the two combinations tested. The FBS - Canada FDA approved was chosen because of a much better transparency of the medium during cell maintenance.

Table 18.I: Effect of combinations of culture medium and serum on SHE cell growth

	Cell growth [a] (n° cells/dish)
MEM + FBS-Canada FDA approved	3.5×10^6
MEM + FBS-EC approved	3.4×10^6

a: culture dishes: 100 x 20mm. Average of 3 experiments. (RDS < 10%).

18.2 pH of Culture Medium

As suggested in the LeBoeuf's protocol *(Kerckaert G.A. et al., 1996)*, a decrease in the pH value up to 6.7 (10% of CO_2, 20% of serum) should result in a 5-10-fold increase in transformation frequency and in overall improvements of cell conditions.

Therefore, two concurrent experiments were carried out in order to check eventual differences in cell survival when cells are grown at basic or acid pH:

- pH = 7.4, initial value of MEM culture medium;
- pH = 6.7, obtained by acidification of MEM culture medium with HCl.

Table 18.II shows that under the adopted experimental conditions (10% of CO_2 and 20% of FBS) the cell viability at pH = 7.4 was 10-fold higher than the corresponding value at pH = 6.7.

Table 18.II: SHE cell growth at basic and acid pH of culture medium

	Cell growth [a] (n° cells/dish)
pH = 7.4	3.3×10^6
pH = 6.7	2.9×10^5

a: growing time: 5 days . Change of medium: twice. Culture dishes: 100 x 20mm. Average of 3 experiments. (RDS < 10%).

18.3 Culture Dishes

A successful performance of the CFE test is often depending on the substrate (e.g. type of plastic) to which cells adhere. Therefore, CFE experiments (Figure 6.1) were carried out seeding SHE cells on culture dishes manufactured by two different companies, such FALCON and NUNC (Table 18.III).

Unexposed cells were grown in culture medium for 24 h and 72 h. The medium was supplemented with 20% of the previously selected serum (Table 18.I), the cells were incubated under 10% CO_2 in the atmosphere, and the pH was maintained at pH = 7.4.

Table 18.III shows that the best cell growth was obtained when FALCON culture dishes, but not NUNC, were used under our experimental conditions.

The mean of colonies/plate obtained after 24- and 72-hour exposure (51 and 47, respectively) was in agreement with the requirements of the assay *(Kerckaert G.A. et al., 1996)*. Moreover, the dividing activity of the target cells was confirmed by the mean of colonies: 9 after 24-hour and 3 after 72-hour exposure. Then, since the feeder cells are only able to grow as a monolayer excluding any colony formation, their inactivation was also verified.

Table 18.III: SHE cell growth on two different plastic culture dishes

Exposure	**Culture dishes as control for feeder and target cells**	**End of the experiment [a] (mean of colonies/plate)**	
		FALCON	**NUNC**
24 h [b]	**Feeder and target cells**	51	0
	Only feeder cells seeded [c]	No colony formation. Cell growth as monolayer	0
	Only target cells seeded [c]	9	0
72 h [b]	**Feeder and target cells**	47	0
	Only feeder cells seeded [c]	No colony formation. Cell growth as monolayer	0
	Only target cells seeded [c]	3	0

a: average of 3 experiments. b: 21 culture dishes/exposure time. c: 2 culture dishes/exposure time.

19 In Vitro Cellular Biotransformation and Interaction of Metal Compounds in Culture Medium and Cellular Lysate

The present section reports the results obtained from High Field NMR analysis (Section 10) on the Balb/3T3 cell line concerning:

a) the characterisation of the chemical and biochemical behaviour of 32 metal compounds in culture media;
b) the identification of the chemical form of 4 metal species in the lysate of cells exposed to μM concentrations of individual compounds.

This study was carried out in collaboration with the University of Torino and the Bioindustry Park of Ivrea (Italy).

19.1 Culture Media

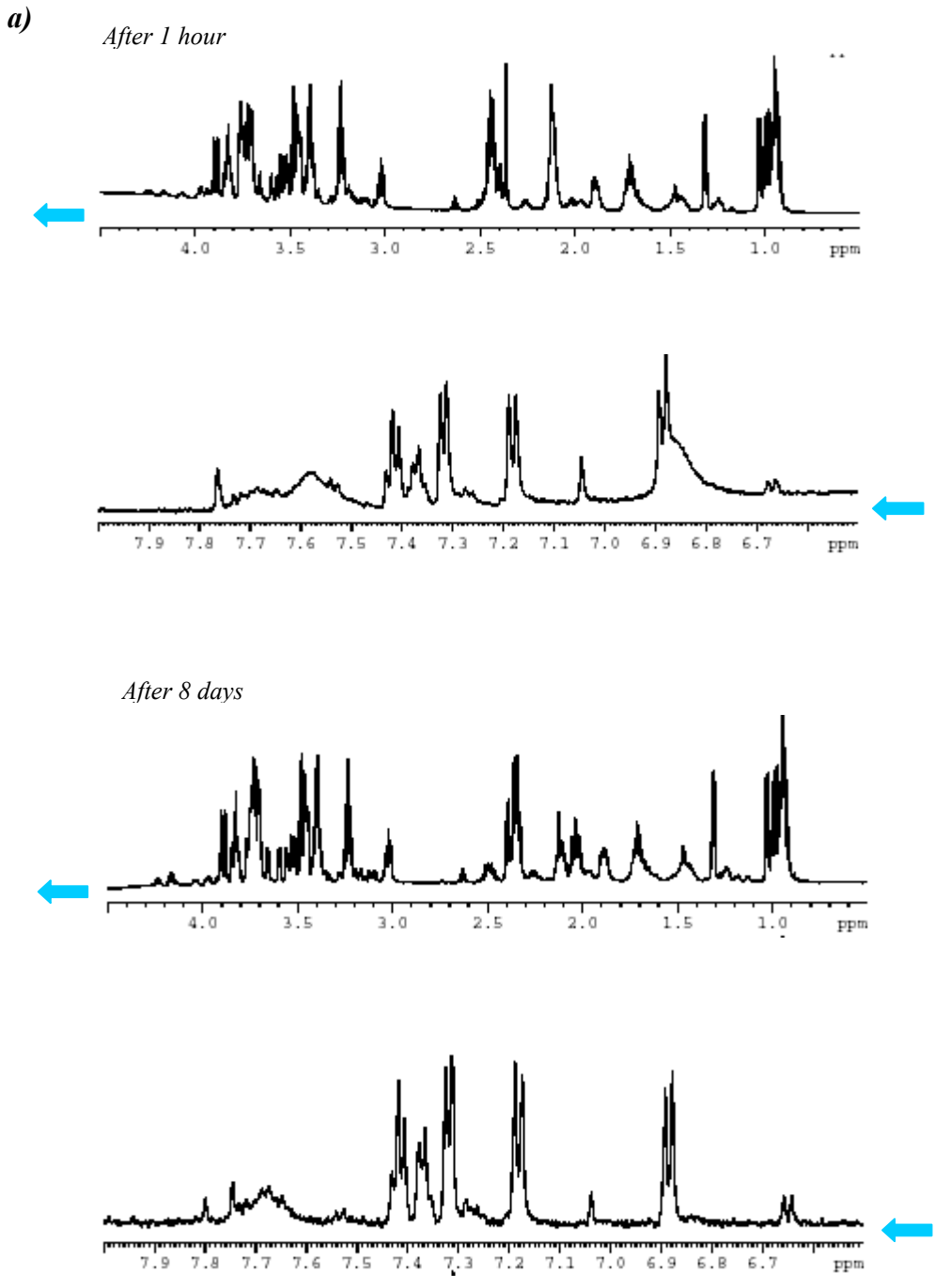
The results concern the comparison between the NMR spectra obtained from culture medium containing a metal compound and the NMR signals produced by non-treated culture medium (control), under the same experimental and technical conditions. Therefore, if a variation between control and treatment exists, it is analysed in order to understand which proton of which molecule generates this different signal. The analyses were carried out on four culture media (DMEM Low-Glucose, DMEM High-Glucose, MEM, RPMI 1640) normally used for culturing cell lines in our laboratory. Each sample was tested as containing or not containing the related serum (Table 10.I). The medium composition was taken into account for the analytical investigation.

In order to exclude any variation of the signals due to either the added reagents or biotransformation of the culture medium, before the analysis each culture medium (added with the proper reagents) was monitored over different periods of time (1 and 12 hours; 3, 5 and 8 days) both in absence and in presence of serum.

The spectra obtained showed that no variation of the NMR signals related to the four culture media tested was found over the monitored periods of time. Therefore, no

degradation of the medium occured (Figure 19.1).

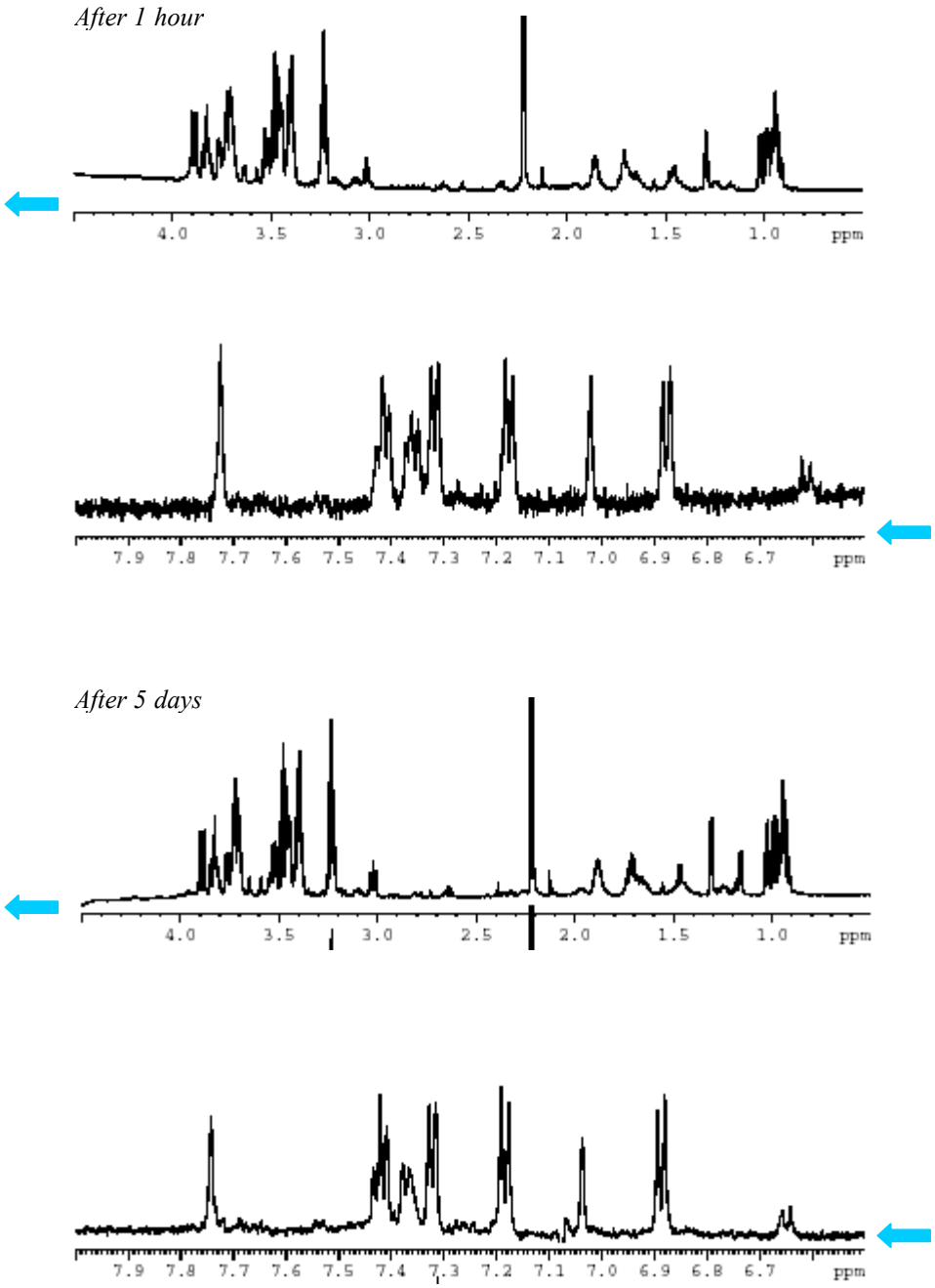
Figure 19.1: NMR spectra of culture media [a]:
a) after 1 h and 8 days, DMEM Low-Glucose, serum free;
b) after 1 h and 5 days, MEM, serum free



a: arrows indicate a continuation of the spectra that were split only for graphical representation problems

Figure 19.1: continued

b)



Once the compositions as well as the NMR spectra of non-treated culture media were known, it was possible to identify all NMR signals of each sample in order to proceed with the comparison of the spectra between control and treated culture media.

Table 19.I reports 32 metal compounds selected on the basis of previously reported cytotoxicity studies (Section 13). They were tested in the four selected culture media with and without serum.

Identical results were detected when an individual treated culture medium was analysed in presence and in absence of the corresponding serum.

Furthermore, before performing the analysis, it was taken care to verify that the pH of each solution was similar to the value of the corresponding non-treated culture medium. This similarity was confirmed for all culture media tested (data not shown).

Table 19.I: Effect of the addition of metal compounds [a] to Low- or High-Glucose DMEM, MEM and RPMI 1640 [b] on NMR signals

Effect			
None	**Loss of the His signal**	**Loss of the His and Tyr signals**	**Enlargement of the spectrum**
$NaAsO_2$	$CoCl_2 \cdot 6H_2O$	$AuCl_3$	$CoCl_2 \cdot 6H_2O$
$(CH_3)_2AsNaO_2 \cdot 3H_2O$	$MnSO_4 \cdot 5H_2O$	$CuSO_4 \cdot 5H_2O$	$Cr(NO_3)_3 \cdot 9H_2O$
$(CH_3)_3AsCH_2COO^-$	$NiCl_2$		$CuSO_4 \cdot 5H_2O$
$Bi(NO_3)_3 \cdot 5H_2O$	$(NH_4)_2PtCl_6$		$NiCl_2$
$CdCl_2 \cdot H_2O$	$(NH_4)_2PtCl_4$		
$CdMoO_4$	$PtCl_4$		
$Na_2CrO_4 \cdot 4H_2O$	$PtCl_2$		
$Ga(NO_3)_3 \cdot 6H_2O$	*cis*-Pt		
$HgCl_2$			
CH_3HgCl			
$(NH_4)_2IrCl_6$			
$(NH_4)_3IrCl_6 \cdot H_2O$			
K_2MoO_4			
carbo-Pt			
$K_2TeO_3 \cdot H_2O$			
K_2TiO_3			
$(C_5H_5)_2TiCl_2$			
$(NH_4)_2[TiO(C_2O_4)_2] \cdot H_2O$			
$NaVO_3 \cdot H_2O$			
$(C_5H_5)_2VCl_2$			
K_2WO_4			

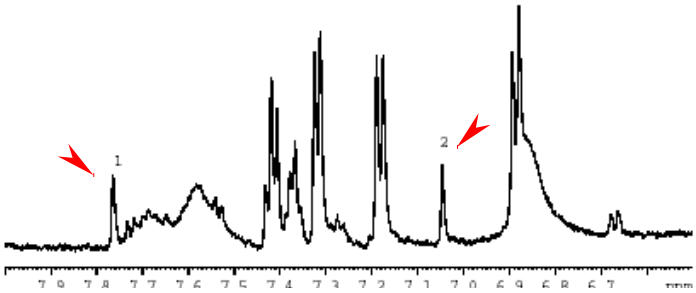
a: As-, Cr- and Pt-compounds, $CdCl_2 \cdot H_2O$ and $NaVO_3 \cdot H_2O$ were analysed at 5 mM. $AuCl_3$, cis-Pt and $NiCl_2$ were tested at 1 mM. The other metal compounds were tested at 100 µM. b: each culture medium was analysed with and without serum.

The results showed no variations of the signals detected in the NMR spectra of the culture media treated with 21 metal compounds (first column of Table 19.I) that were considered "inert" in relation to interactions with components of culture medium. However, in the case of $(C_5H_5)_2TiCl_2$ a gradual, but not complete, loss of the signals corresponding to the protons of the two cyclopentadienilic rings was detected. Only in the RPMI 1640 culture medium the signals were totally lost. It is inferred that the loss of these specific signals for the titanocene molecule corresponds to a gradual disappearance of the metal compound in the solution.

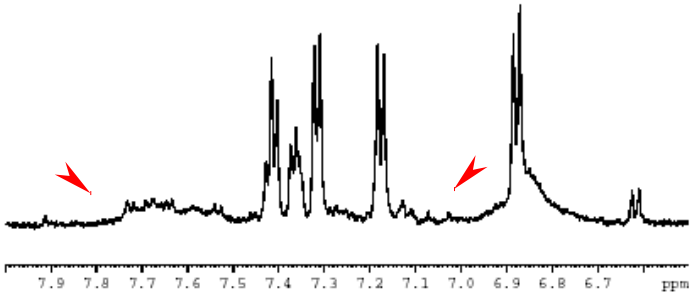
As shown in Table 19.I, loss of the histidine signal for 8 metal compounds as well as the concurrent loss of the histidine and the tyrosine signals for $AuCl_3$ and $CuSO_4 \cdot 5H_2O$ was detected. This is an index of: a) the formation of a precipitate; b) the presence of a paramagnetic species; c) the coexistence of both previous situations. In this context, the only exception was $CoCl_2 \cdot 6H_2O$. This metal compound did not precipitate, however it showed paramagnetic properties so as $Cr(NO_3)_3 \cdot 9H_2O$, $CuSO_4 \cdot 5H_2O$ and $NiCl_2$. These properties caused an enlargement of the detected signals that usually can disturb the NMR analysis. In fact, this line broadening was particularly intense for Cr(III), and thus no comparison between the NMR spectra of control and treated medium was possible (data not shown).

Moreover, the loss of a specific signal followed a kinetic that was different for each metal compound. This was particularly evident for Pt-compounds, namely, *cis*-Pt, $(NH_4)_2PtCl_6$, $(NH_4)_2PtCl_4$, $PtCl_4$ and $PtCl_2$, with the only exception of carbo-Pt, which showed no interaction with the components of the culture media tested. As showed in Figure 19.2, the loss of the two peaks at 7.03 and 7.74 ppm (see arrows) identifying the His signal was complete after 1 hour for $PtCl_4$, 16 hours for $(NH_4)_2PtCl_6$ and $(NH_4)_2PtCl_4$, 3 days for $PtCl_2$, and after 5 days for *cis*-Pt.

Figure 19.2: Loss of His signal of culture media as result of addition of Pt-compounds

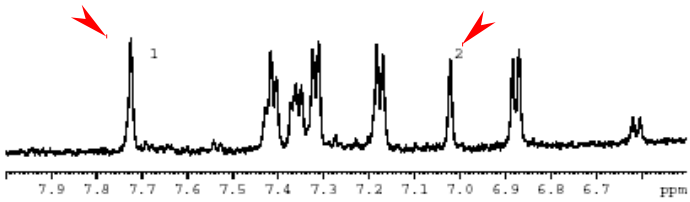


DMEM after 1 hour

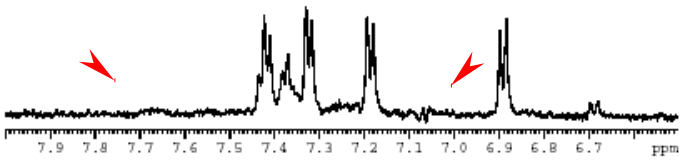


DMEM + cis-Pt after 5 days

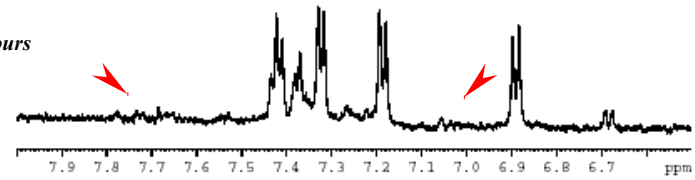
MEM serum free after 1 hour



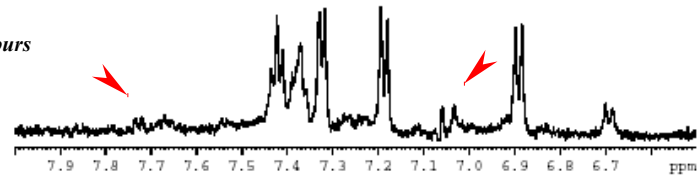
MEM s.f. + $PtCl_4$ after 1 hour



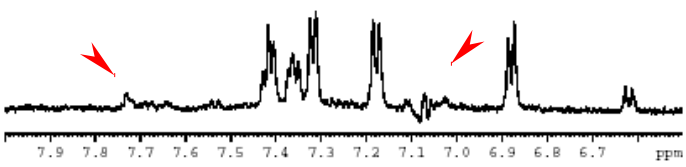
MEM s.f. + $(NH_4)_2PtCl_6$ after 16 hours



MEM s.f. + $(NH_4)_2PtCl_4$ after 16 hours



MEM s.f. + $PtCl_2$ after 3 days

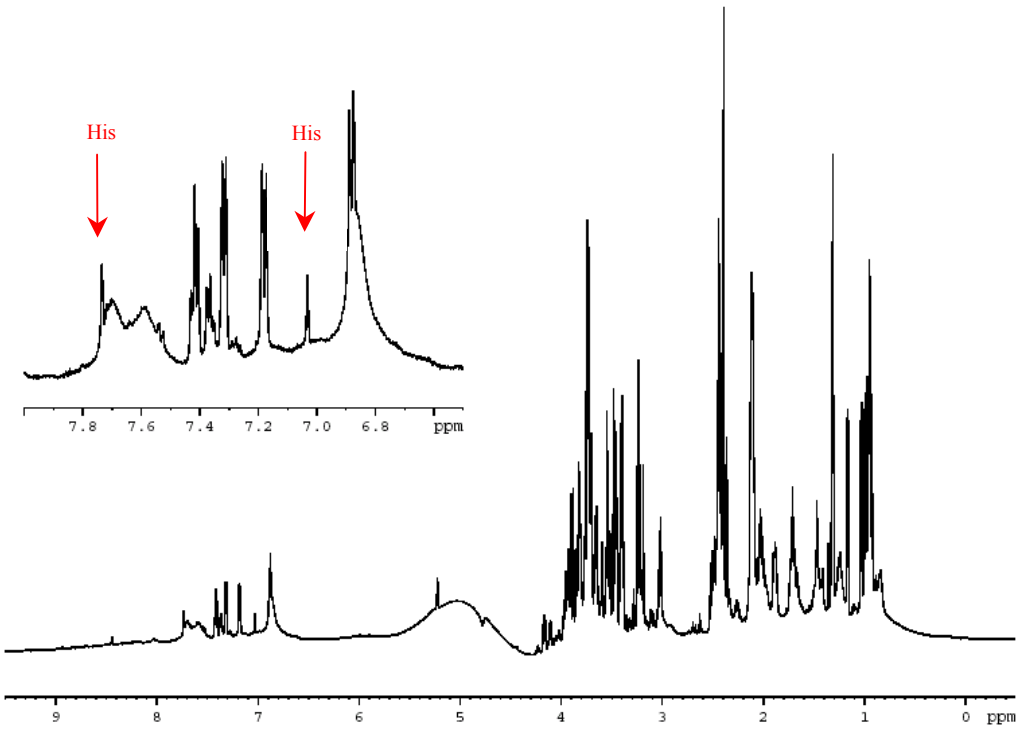


As consequence of the loss of the His signal, the presence of a white precipitate in each Pt solution was observed at Pt concentrations of the order of 5 mM (1 mM for *cis*-Pt only). This product was supposed to be a Pt-His complex and was found insoluble in H_2O and inorganic solvents, such acetone, methanol, acetonitrile, but soluble in 0.1% trifluoroacetic acid. An accurate characterisation was made applying three different techniques (data not shown):

a) CP-MAS (Cross Polarisation – Magic Angle Spinning), which characterised the species as solid sample;
b) TOF-MS (Time Of Flight - Mass Spectroscopy) that determined the 1:2 stechiometric ratio for the supposed Pt-His complex;
c) ^{1}H-NMR that definitively confirmed not only the existence of a Pt-His complex but also its 1:2 stechiometric ratio.

Recent experiments were carried out exposing 72 h the Balb/3T3 cells to 100 µM $(NH_4)_2PtCl_6$. Interestingly, the NMR spectrum of the treated culture medium (DMEM Low-Glucose) after exposure to cells indicated a persistence of the two peaks for the His molecule (see arrows in Figure 19.3).

Figure 19.3: NMR spectrum of DMEM Low-Glucose after contact with cells exposed 72 h to 100 µM $(NH_4)_2PtCl_6$



19.2 Cellular Lysates

The results reported in this section refer to NMR analysis of cytosolic extracts (Section 10) of the Balb/3T3 cells exposed 72 h to:

- $NaAsO_2$, 1.5 µM;
- $(NH_4)_2PtCl_6$, 3.7 µM;
- $Na_2CrO_4 \cdot 4H_2O$, 3.6 µM;
- $NaVO_3 \cdot H_2O$, 4.7 µM.

The concentration, at which each selected metal compound was tested, corresponds to the IC_{50} value previously reported for these species (Table 13.III).

Before carrying out the analysis on the treated lysates (Figure 19.4), the NMR spectra of three controls prepared in different days (cellular lysates derived from non-treated cells) were compared. The analysis revealed no difference among the three spectra and consequently it confirmed the reproducibility of the applied system (data not shown).

Figure 19.4: NMR spectra of cellular lysates of Balb/3T3 cells exposed to a) As-, b) Cr-, c) Pt- and d) V-compounds for 72 h

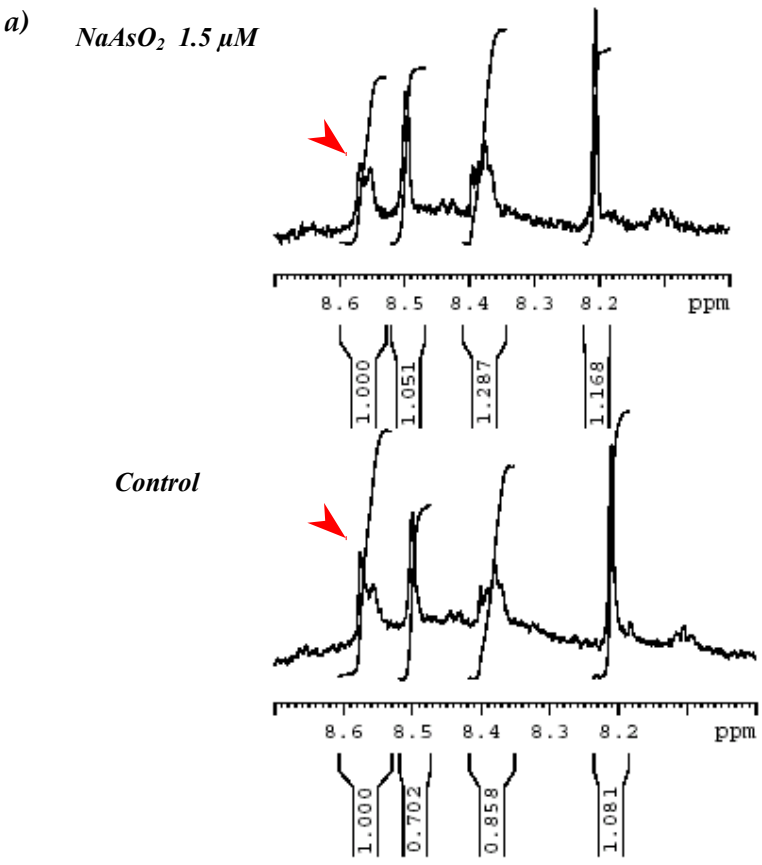
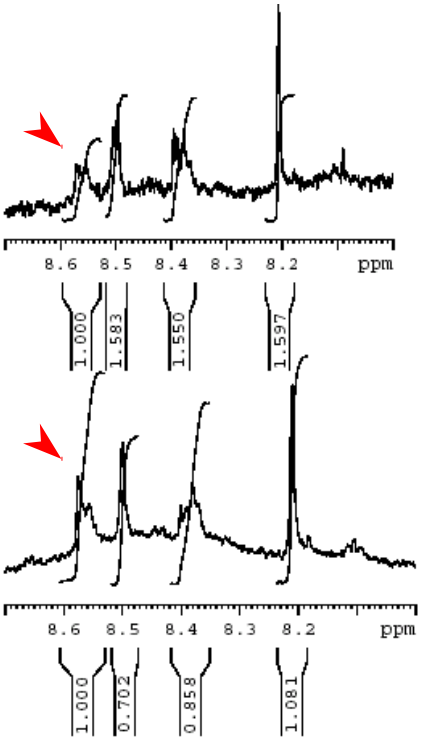


Figure 19.4: Continued

b)

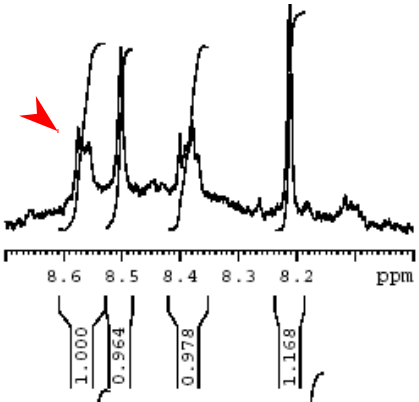
$Na_2CrO_4 \cdot 4H_2O$ 3.6 µM



Control

c)

$(NH_4)_2PtCl_6$ 3.7 µM



Control

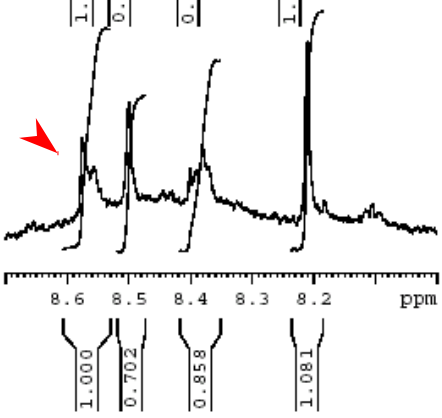
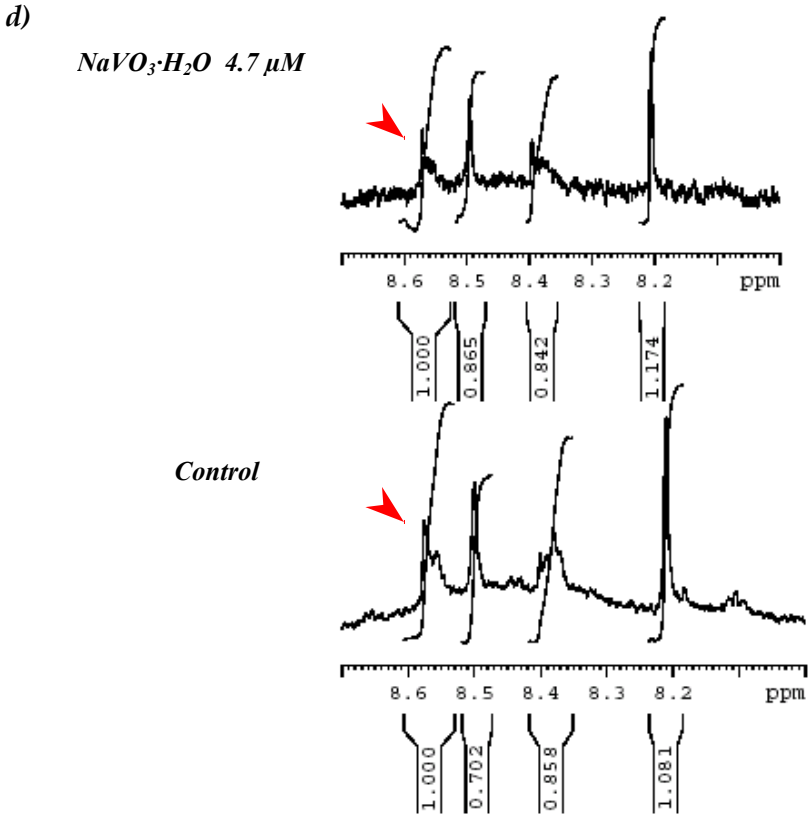


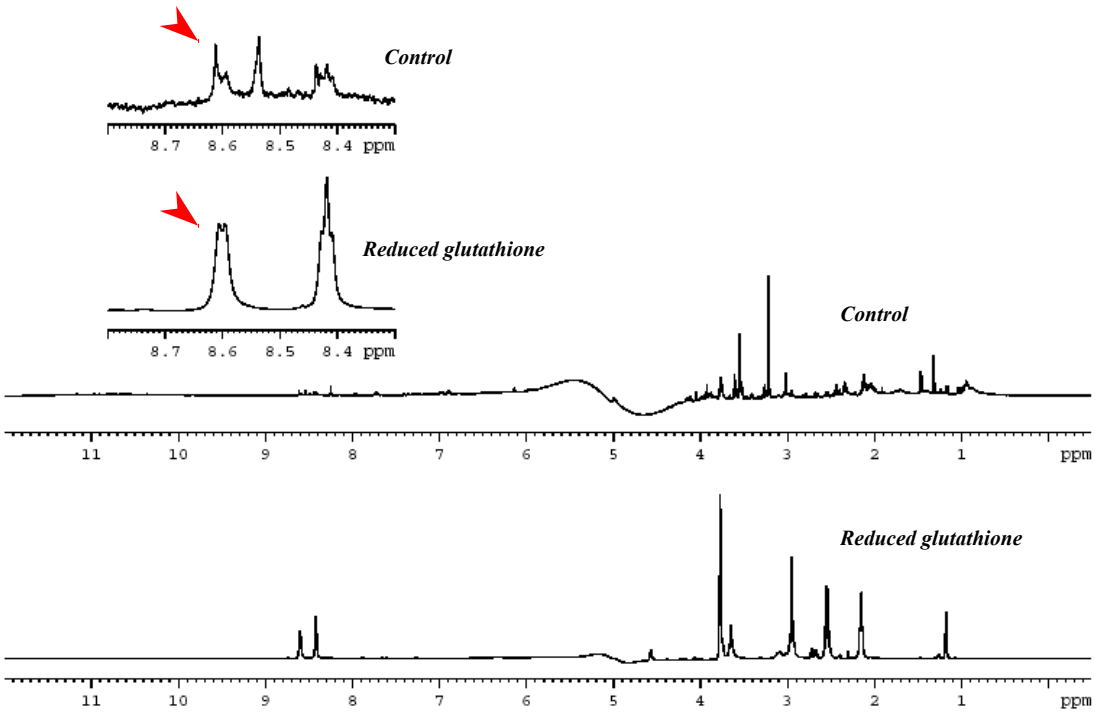
Figure 19.4: Continued



Compared to the control, a change in the signal at 8.58 ppm was identified for $NaAsO_2$, $Na_2CrO_4{\cdot}4H_2O$ and $(NH_4)_2PtCl_6$, whereas no variation in the spectrum of $NaVO_3{\cdot}H_2O$ was detected (see arrows in Figure 19.4*a-d*). In the first three treatments this signal at 8.58 ppm appeared with a modified shape, more symmetric in comparison with the control (see $NaAsO_2$, where this change is particularly evident). Furthermore, the ratio between the areas of the identified signal to the areas of its chemical environment was decreased. This confirms a decrease of the protons that generate the signal. Consequently, it is to suppose that after cell exposure to $NaAsO_2$, $Na_2CrO_4{\cdot}4H_2O$ and $(NH_4)_2PtCl_6$ a chemical species disappears.

Further analysis indicated that this modified signal at 8.58 ppm corresponds to the reduced glutathione molecule (see arrows in Figure 19.5).

Figure 19.5: Modified signal at 8.58 ppm identified as the signal of reduced glutathione



We can suppose that two overlapped signals form the asymmetric signal at 8.58 ppm evident in the control (Figure 19.5). After exposure to As-, Cr- and Pt-compounds, one of these two signals, identified as the reduced glutathione, remains unchanged in shape and position. On the other hand, the second signal is modified because it disappears in the spectrum detected after treatment.

DISCUSSION

Hereafter is reported the discussion of the results achieved in the present reasearch in relation to the planned objectives (see page 11).

♦ **Check of chemical purity.** A key factor in the study of the carcinogenic potential of metal compounds concerns their chemical purity. Elemental impurities of metal compounds tested (particularly those with same chemical affinity for the metal under study) could lead to artefacts regarding the cytotoxic and transforming response. Then, we must be sure about the degree of chemical purity of metal salts used in our experiments.

The analysis by ICP-MS of metal impurities of the concentrated 10^{-2} M $(NH_4)_2PtCl_6$ solution used in transformation assay is an example (Table 12.II). In such solution elemental impurities were almost present at µg/l level. At the highest Pt concentration tested (100 µM) impurities added into the culture medium with Pt solution were calculated in the order of 10^{-9} to 10^{-13} M. These amounts were judged not able to induce either cytotoxic effects or morphological transformation under our experimental conditions. Similar results have been recently obtained for other salts of Cd, Cr, Hg, Ir, Pt and V that gave positive transforming response in the Balb/3T3 cells (data not shown).

As in the present work, studies on the carcinogenic potential of metals must be carried out using metal species of the best existing chemical purity (pure/ultrapure grade). Our findings confirm the very good chemical purity of such commercially available metal compounds making them suitable to avoid *in vitro* artefacts concerning the biological response.

♦ **Standardisation and optimisation of the Balb/3T3 assay.** The transferability and reproducibility of test protocols among different laboratories are basic aspects for prevalidation/validation studies of *in vitro* toxicity testing *(Balls M. et al., 1990)*.

In this context, the quality check of culture media and sera was investigated on the basis of the cell survival, trypsinisation time, transparency of the culture media and sera

tested as well as change in cell morphology. For this purpose CFE experiments were set up using specific combinations of the analysed reagents. The products of EURO CLONE and HY CLONE for culture medium and serum, respectively, were identified as products to ensure the best conditions of cell growth (Table 12.I).

Influence of the source of cells on the performance of the assay was assessed using Balb/3T3 cell line from two different laboratories. These two pools were analysed for growth, viability and cytotoxic response in order to choose the cells with the best features in relation to the requirements of the assay (Table 12.IV). Quantitative differences were observed between the two cell pools, confirming the importance of the source of the cell line as a factor of variability.

The pH of culture medium was considered with particular attention. Any variation may deeply affect cell culture conditions and ultimately cell growth and biological response.

The experiment set up in order to investigate effects of the pH value of culture medium on cell growth showed intriguing results, particularly considering the pH estimate without cells (Table 12.V). Under our experimental conditions, the pH of the starting medium was about 7.4 – 7.5. During the working time it tended to become 7.6 – 7.7 without changing anymore. These conditions were judged satisfactory for our experiments.

Thawing process is a basic aspect to preserve unchanged the cellular features. Two different methods were compared on the basis of the best cell growth obtained some days after the thawing (Table 12.VI). Surprisingly, different results were obtained in cell survival, one procedure (standard method) giving a number of survived cells/dish 3-fold higher than the second procedure (specific method).

In order to highlight eventual differences in sensitivity in the measurements of endpoints, two different cytotoxicity tests, CFE and NRU, were carried out on the Balb/3T3 cells exposed to $(NH_4)_2PtCl_6$. CFE was the method of choice (Table 12.VII). The higher cell viability of the NRU test compared to the CFE assay is probably due to the further 7-day incubation after treatment in the case of the CFE test that may allow the metal compound to act longer on the cellular target.

The intralaboratory reproducibility of the CFE test was verified and confirmed by estimating the mean variations of a CFE experiment carried out by four operators, which worked over three different days (Table 12.VIII). Although the experiments were performed in the same experimental conditions, the mean variations for the same operator were greater than those obtained for different operators. A reasonable explanation is that fortuitous and unforeseeable variations in cell culture conditions as well as in cell handling can occur from time to time.

♦ **Cytotoxicity and morphological transformation of metal compounds in Balb/3T3 cells.** The strategy adopted in the present study for the determination of the carcinogenic potential of metal compounds in Balb/3T3 cell line involved four steps:
i) determination of cytotoxicity at a fixed concentration of metal exposure;
ii) setting of dose-response relationships for those compounds identified as relevant from step (*i*), in order to establish the corresponding IC_{50} values and suitable range of concentrations to be used in the third step;
iii) determination of concurrent cytotoxicity and morphological neoplastic transformation of selected metal compounds from step (*ii*);
iv) mechanistic studies on metal compounds identified transforming in Balb/3T3 cells.

The experiments related to the first step involved the evaluation of the cytotoxicity of 65 metal compounds (Table 13.I). This permitted to establish a ranking of metal compounds as three groups according to their degree of cytotoxic response. The group I included 30 metal compounds (no observable or little cytotoxic effect on cells: CFE not lower than 80% of the control). In this group the only essential elements to humans that do not significantly affect CFE are selenium, as Se(IV) and Se(VI), and chromium, as Cr(III).

The group II included 13 metal compounds (CFE between 80% and 30% of the control). The ranking of cytotoxicity expressed as elements is: Pt(II) > Br(V) > Te(VI) > Zn(II) > Tiorg(IV) > Be(II) > Pb(II) > Rh(III) > Ni(II) > Th(IV) > U(VI) > W(VI) > Sn(II). The only essential element in this group is Zn(II) that caused a CFE inhibitory effect of the order of 50%.

The group III included 22 metal compounds (strong or complete CFE inhibition): Ag(I) = As(III) = As(V) = Bi(III) = Cd(II) = Cd(II)+Mo(VI) = Cr(VI) = Ga(III) = Hg(II)

= MeHg(II) = Mn(II) = Pt(IV) = Te(IV) = Vorg(V) = V(V) = V(IV) > Au(III) > Ir(IV) > Ir(III) > Cu(II) > Co(II).

Essential trace metals of this group include Co(II), Cu(II) and Mn(II). In addition, As(III)-, Cd(II)-, Cr(VI)- and V(V)-compounds have already been proved to be strongly cytotoxic in the Balb/3T3 *(Bertolero F. et al., et al., 1987a; Bertolero F. et al., 1987b; Sabbioni E. et al., 1993)* and in the Syrian Hamster Embryo (SHE) cell lines *(DiPaolo J.A. and Casto B.C., 1979; Elias Z. et al., 1989; Rivedal E. and Sanner T., 1981; Rivedal E. et al., 1990)*. The strong cytotoxic effect induced in Balb/3T3 by Ag(I), Au(III), Co(II), Cu(II), Hg(II), MeHg(II), Mn(II), Pt(IV) and Te(IV) represents a new finding (Table 13.I).

Metal compounds of groups III and II were considered of "first priority" for the subsequent study concerning the setting of dose-effect relationships (Section 13.2). Moreover, an interesting finding is the dependence of the cytotoxicity on the chemical form of individual metals tested (As-, Br-, Cr-, Hg-, Ir-, Pt-, Te-, Ti- and V-compounds; Table 13.III and Figure 13.2). This suggests that the Balb/3T3 system is a valuable *in vitro* model in relation to metal speciation (Section 3.2), which is a key factor in assessing metal toxicity in mammals *(Sabbioni E. et al., 1985)*.

Caution, however, must be used in the interpretation of the classification of metal compounds in three groups on the basis of their cytotoxic response. This ranking is arbitrarily and perhaps it cannot be used for predicting the *in vitro* potential carcinogenic activity according to the cytotoxic effect of tested metal species. The classification must be intended as a rational basis of a working methodology that takes advantage of the Balb/3T3 sensitivity to tumour agents, and makes possible the use of the two-stage protocol (concurrent cytotoxicity and morphological transformation assay). Such protocol imposes a preliminary toxicity test based on the CFE in order to establish the optimal concentration levels for the transforming assay *(IARC/NCI/EPA Working-Group, 1985)*. In addition, the screening study has been carried out at a relatively high concentration of metal exposure (100 μM). Due to the two-stage nature of the Balb/3T3 assay, for metal compounds giving a negative cytotoxic response at such concentration a subsequent transformation assay at lower concentrations would not make sense. Thus, it was reasonable to use the cytotoxicity data in suggesting a prioritisation of metal compounds to be tested for their potential transforming activity.

Furthermore, metal-induced cytotoxicity and transforming processes are not necessarily related events. This is confirmed by the cases of Ag(I), Au(III), Bi(III), Co(II), Cu(II), Ga(III), Hg(II), Ir(III), Mn(II), Te(IV), Vorg(V) and V(IV) that are strong cytotoxic, but not transforming, in the Balb/3T3 cells (group III, Table 13.I). For what it concerns metals classified as group I and II, preliminary experiments have shown no transforming activity for 13 metal compounds of group I and II, to which the Balb/3T3 cells were exposed at concentrations from 100 μM to 1000 μM *(personal communication)*.

The setting of dose-effect relationships was carried out on 35 metal species at a wide range of concentrations from 0.01 μM to 5000 μM, in order to establish dose-effect curves (Section 13.2). This allowed the calculation of the 50% inhibition concentration (IC_{50}) values, a key parameter for selecting the concentrations of the metals to be used in the third step of the study (concurrent cytotoxicity and morphological transformation assay). From the dose-effect curves IC_{50} values ranging from 0.32 μM for CH_3HgCl to 8380 μM for KBr were derived (Tables 13.II and 13.III).

Most of the metal species considered were tested in order to take into account the aspect of speciation. In fact, it is well recognised nowadays that humans are exposed to various physico-chemical forms of inorganic and organometallic species of a same element, which can have different metabolic pathways that can lead to different interactions with cells and ultimately to different toxicological effects *(Sabbioni E. et al., 1985)*. Such "chemical species" (defined by IUPAC terminology as specific form of a chemical element defined according to its molecular, complex, electronic or nuclear structure; *Templeton D.M. et al., 2000*) can carry widely different health effects playing a fundamental role in setting health protection standards *(Sabbioni E. et al., 1991a)*.

In the present work, the derivation of IC_{50} values concerning metal species with different oxidation states, either as inorganic (cationic/anionic) or organometallic forms of As, Br, Cr, Hg, Ir, Pt, Te, Ti, V (Table 13.III) confirms the validity of this cellular model in relation to the aspect of speciation *(Bertolero F. et al., 1987a; Sabbioni E. et al., 1987; Sabbioni E. et al., 1991b)*. Figure 13.2 gives graphically strong evidence of the influence of the oxidation state of metal species (cationic or anionic forms, Cr^{3+} and CrO_4^{2-}, VO^{2+} and VO^{3-}, or only anionic forms, AsO^{2-} and AsO_4^{3-}, Br^{-1} and BrO^{3-}, $PtCl_4^{2-}$ and $PtCl_6^{2-}$, TeO_3^{2-} and TeO_4^{2-}) on their cytotoxicity.

In addition, the results show that the inorganic or organometallic nature of an individual metal affects its cytotoxicity. Examples concern $NaAsO_2$ and As-betaine, MMA and DMA; $HgCl_2$ and CH_3HgCl; $(NH_4)_2[TiO(C_2O_4)_2]\cdot H_2O$ and $(C_5H_5)_2TiCl_2$; $VOSO_4\cdot 5H_2O$ and $(C_2H_5)_2VCl_2$ (Table 13.III and Figure 13.2). Some of these findings are in agreement with previous *in vivo* and/or *in vitro* observations. Inorganic trivalent and, to a less extent, pentavalent arsenic species are toxic and transforming in the Balb/3T3 cell line *in vitro*, whereas organoarsenic compounds are not toxic *(Bertolero F. et al., 1987a; Harada M., 1995)*. Hexavalent soluble Cr-compounds appear 100 to 1000-fold more cytotoxic to human fibroblasts than trivalent Cr-compounds *(Katz S.A. and Salem H., 1993)*. Methylmercury, but not inorganic mercury, induces neurological damage in the PC12 cell line *(Cocco B., 1999)*. Pentavanadate ions are cytotoxic and transforming in the Balb/3T3 mouse fibroblasts compared to tetravanadate ions that are much less toxic and not transforming *(Sabbioni E. et al., 1991a)*. The study of some chemical forms of Br, Hg, Ir, Pt, Te, Ti and V in the Balb/3T3 cell line further enriched the database concerning the effect of metal speciation on the cytotoxic response.

The data obtained from the cytotoxicity studies were also analysed from a statistical point of view. In addition to the evaluation of the reproducibility of the assay by the ANOVA approach, two *post hoc* tests, the Dunnet and the Tukey tests, were applied (Section 11). Using the Dunnett test, each treatment was compared to the control, the threshold for significance being at $p<0.05$, $p<0.01$ and $p<0.001$ for treatment concentrations. Moreover, the application of the Tukey test provided further evidence of the influence of metal speciation on the cytotoxic response confirming significant differences ($p<0.01$, $p<0.001$) between the different chemical forms of As (except for DMA and MMA), Br, Cr, Hg, Ir, Pt, Te, Ti and V.

Other interesting considerations related to the work on the cytotoxicity in Balb/3T3 can be drawn from the two examples of combined mixtures of metal compounds (Section 13.5) as well as other two examples of different chemical species of Pt- and As-compounds (Sections 13.3 and 13.4).

Concerning the study on combined mixtures of metal compounds, the cytotoxic potential of Pt(IV) was effectively proved to be the toxic species when tested in presence of Rh(III)- or Pd(IV)-compounds (Table 13.VIII), confirming the data obtained from individual metal species (Table 13.I). This finding suggests the need to

assess the health risk of platinum as "newer" potential environmental pollutant *(Murdoch R.D. and Pepys J., 1987; Pietra R. et al., 1994)*.

From the experiments on the cytotoxicity of "hard metals" (Table 13.IX, Co alone or in presence of Mo, Ti, W) synergistic or antagonistic effects can be excluded (cell survival obtained from the exposure to Co similar to that observed in combination with the other metals). However, when vanadium(V) was added to the mixture a dramatic decrease of cell viability leading to a complete growth inhibition was observed.

This confirms that Balb/3T3 is a suitable model not only to assess the cytotoxic effect of individual compounds but also to study complicated aspects such as multiple exposures as in the case of "hard metals" (Co, Mo, Ti, V, W) *(Sabbioni E. et al., 1994)*.

Concerning the study on two groups of metals, Pt- and As-compounds, it is interesting to discuss the results on their cytotoxicity (Sections 13.3 and 13.4) in relation to the data of their carcinogenic potential (Sections 14.2 and 14.3) and metabolic studies (Sections 15.1 and 15.2).

Pt-compounds. As shown in Figures 13.3-13.5 the comparison of cytotoxicity has regarded pairs of metal forms, namely, $(NH_4)_2PtCl_4$ and $(NH_4)_2PtCl_6$ (inorganic anionic species); $PtCl_2$ and $PtCl_4$ (inorganic cationic forms); *cis*-Pt (inorganically complexed ion); and carbo-Pt (organoplatinum compound). Platinum in oxidation state +4 as anionic form was stronger cytotoxic than the corresponding anionic form +2 (Figure 13.3). This finding was also confirmed by the data reported in Table 13.V, with the only exception of $Na_2PtI_6 \cdot 6H_2O$. A hypothesis is that the PtI_6^{2-} complex may have a so high stability in culture medium to influence its bioavailability to cells and ultimately its cytotoxicity.

However, Table 13.IV indicates a still more complicated situation. The derived IC_{50} values showed a degree of cytotoxicity about 4-fold higher for Pt(II)-compounds as organic form (carbo-Pt), inorganically complexed ion (*cis*-Pt) and cationic form ($PtCl_2$) compared to the anionic Pt(IV)-species.

These findings slightly differed from the results of morphological neoplastic transformation studies (Table 14.II). Indeed, the derived rankings of cytotoxic (*i*) and carcinogenic potential (*ii*) allow the situation to be better focused:

i) $PtCl_2 \geq$ *cis*-Pt > carbo-Pt > $PtCl_4 \geq (NH_4)_2PtCl_6 >> (NH_4)_2PtCl_4$

ii) *cis*-Pt >> carbo-Pt > $PtCl_2 >> PtCl_4 > (NH_4)_2PtCl_6 >> (NH_4)_2PtCl_4$

A comparable level of cytotoxicity and carcinogenic potential was evident for Pt(IV)-compounds, where the cationic form showed an effect slightly stronger than the anionic species. On the other hand, the cytotoxicity values of $PtCl_2$, *cis*-Pt and carbo-Pt were similar (0.7 μM < IC_{50} < 1.8 μM), whereas *cis*-Pt was the Pt-compound showing the highest transformation frequency ($29.1x10^{-4}$ for the comparison at 0.7 μM) (Table 14.II). The only exception was the anionic form $(NH_4)_2PtCl_4$, a Pt(II)-species that seems to have a low degree of toxicity and to be unable to induce carcinogenic effects under our experimental conditions.

These findings show again how speciation plays a fundamental role in determining the biological response.

In this context, studies on Pt-uptake from different chemical forms of platinum tested (Table 15.I) represent an important contribute to explain the differences of the cytotoxic/transforming activity. The incorporation of platinum into cells was different for the different species tested. Thus, the different cytotoxic response could depend on the different bioavailability of platinum to cells or different metabolic pathways.

As-compounds. The results of cytotoxicity studies of this work (Table 13.I and Figure 13.2) are in agreement with *in vivo* observations. Inorganic As-compounds are more toxic than organoarsenic species, the trivalent being more toxic than the pentavalent forms *(Eisler R., 1994)*. However, some As(V)-species, namely, $NaAsF_6$, $KAsF_6$ and $LiAsF_6$ (Table 13.VI), did not induce any cytotoxic effect unlike $Na_2HAsO_4 \cdot 7H_2O$. We can suppose that the AsF_6^- complex may have a so high stability in culture medium that arsenic is no bioavailable (in culture medium or in the cell) to exert its toxic effect.

In this context, interesting results were achieved from metabolic studies. Radiotracers experiments by ^{73}As showed how the speciation can influence the incorporation of metal in the cells. The cellular uptake of As(III) was about 4-fold higher than As(V) (Table 15.II). At intracellular level and at not toxic concentrations As in the cell was mostly cytosolic. At toxic concentrations an obvious shift of the metal on the cellular organelles was found (Table 15.III). This suggests that a saturation of As-binding sites in the cytosol was reached.

Furthermore, the study on cytotoxicity (Table 13.VII) and carcinogenic potential (Table 14.III) of organoarsenic species confirms *in vivo* observations *(Shiomi K., 1994; Shiomi K. et al., 1988; Yamauchi H. et al., 1989)* with a new finding. In fact, the

tetraphenylarsonium chloride hydrate ($(C_6H_5)_4AsCl \cdot H_2O$) differed from the other organoarsenic compounds showing a dose-response curve that reached complete growth inhibition at 100 μM (Table 13.VII) and $5.9x10^{-4}$ transformation frequency at 7 μM (Table 14.III).

The work related to the third step of the proposed strategy involved the determination of concurrent cytotoxicity and carcinogenic potential of metal compounds (Section 14). Among the metal compounds tested in a range of concentration from 0.1 to 700 μM, $NaAsO_2$, $Na_2HAsO_4 \cdot 7H_2O$, $CdCl_2 \cdot 2H_2O$, $Na_2CrO_4 \cdot 4H_2O$, $(NH_4)_2PtCl_6$, and $NaVO_3 \cdot H_2O$, were found transforming (Table 14.I).

Arsenic. Table 14.I shows that concentration at least 4-fold higher was required to induce an equivalent transformation frequency for $Na_2HAsO_4 \cdot 7H_2O$ as compared to $NaAsO_2$ (Photo 14.2). This finding is in agreement with previous observations that proved trivalent inorganic arsenic more cytotoxic and transforming compared to the pentavalent form in the Balb/3T3 cells *(Bertolero F. et al., 1987a)* and in the Syrian hamster embryo cells *(Lee T.C. et al., 1985)*. Interestingly, it has been suggested that the same intracellular chemical form of arsenic is responsible for the effect, independently of the valence state of the inorganic arsenic present in the culture medium, because a reduction process of arsenate to trivalent arsenic occurs in Balb/3T3 *(Bertolero F. et al., 1987a)*. A new finding of the present work was the carcinogenic potential of $NaAsO_2$ that was comparable with $(C_6H_5)_4AsCl \cdot H_2O$, while $Na_2HAsO_4 \cdot 7H_2O$ was quantitatively less transforming than this organoarsenic compound. Once more, an As(III)-species displays its higher toxicity also in the context of organoarsenic compounds.

If the inorganic As-species tested were cytotoxic and transforming, a methylated form of arsenic (As-betaine) was ineffective (Tables 13.III and 13.VII; Tables 14.I and 14.III). This is in agreement with the *in vivo* situation *(Maher W.A., 1985; Shiomi K., 1994)*. Methylated arsenicals are rapidly excreted in urine following administration, probably because of their stability in biological media and low affinity for cellular components. Some reports on the metabolism of As in seafood suggest that orally ingested As-betaine is rapidly excreted in human urine without biotransformation, implying that the human body, like experimental animals, does not retain As-betaine. Therefore, a low retention efficiency of As-betaine by Balb/3T3 cells due to its high stability in biological media and to the lack of intracellular binding with

macromolecules *(Sabbioni E. et al., 1991b)* is in agreement with the findings of the present study as well as with *in vivo* and cell-free *in vitro* studies *(Magnani L., 1988; Vahter M. et al., 1983)*.

Cadmium. A further important confirmation of the reliability of the morphological transformation Balb/3T3 assay was obtained from data related to cadmium (Table 14.I) for which sufficient evidence in humans and animals classify this metal as carcinogenic, independently of the Cd-compound tested *(Oldiges H. et al., 1989)*. Even though the underlying mechanisms are still puzzling, Cd(II) was proved to cause DNA strand breaks and chromosomal aberrations, although these latter are induced at highly cytotoxic concentrations *(Hartwig A., 1995)*. Moreover, the accumulation of cadmium in kidneys without apparent toxic effect is possible due to the formation of a Cd-binding protein (metallothionein), a metal-protein complex with a low molecular weight (~6500 dalton), which can give chronic and irreversible nephrotoxicity. Interestingly, cadmium bound to metallothionein within tissues is toxic when taken up by the proximal tubular cells complex, whereas cadmium chloride at even greater concentrations in proximal tubular cells is not toxic *(Dorian C. et al., 1995)*.

Chromium. In the present study, Cr(VI) has been found transforming in Balb/3T3 cells, while Cr(III) was ineffective (Table 14.I). This finding reflects the *in vivo* situation *(Wiegand H.J. et al., 1984)*. After entering cells across non-specific anion channels Cr(VI) is reduced by enzyme-catalysed reactions or non-enzymatic components such a glutathione. This process leads to Cr(III) that can cause several forms of DNA damage (DNA-Cr(III) adducts or oxidative damage by reactive intermediates) *(Voitkun V. et al., 1998)*. In the present study the experiment on the speciation of chromium in the culture medium after incubation of the Balb/3T3 cells with Cr(VI) confirmed that in the cells Cr(VI) undergoes to a biotransformation to Cr(III) that becomes the predominant chemical form in culture medium (Table 15.IV). Furthermore, our findings confirm that cytotoxicity (Table 13.I and Figure 13.2) and carcinogenic potential (Table 14.I) are strictly related to the chemical form of chromium (transforming activity of Cr(VI) already at 30 μM; no carcinogenic potential of Cr(III) up to 500 μM).

Platinum group. Pt(IV), Pd(IV) and Rh(III) were already mentioned as metals of environmental, occupational and biomedical interest (Section 3.1: Platinum Group Metals). This justified a comparative study concerning their cytotoxicity (Table 13.I)

and transforming activity (Table 14.I). Moreover, the adverse effects in humans after environmental exposure (release from automotive catalytic converters), occupational exposure (allergy and asthma in workers), biomedical use (use as antitumour drugs or as components of alloys in dentistry and orthopaedics) are already known *(Pietra R. et al., 1994)*. Nevertheless, there is little information available on the effect on human health after long-term chronic exposure to low levels of platinum and platinoids.

The present study represents a step forward in the context of toxicological risk assessment of Pt-compounds. For the first time the present study shows an *in vitro* transforming activity of $(NH_4)_2PtCl_6$ in Balb/3T3 (Table 14.I and Photo 14.1), while the corresponding Rh(III) and Pd(IV) salts did not show morphological transformation in the same *in vitro* model (Table 14.I).

Vanadium. A previous study on ammonium metavanadate(V) and vanadyl(IV) sulphate in Balb/3T3 cells demonstrated that V(V) is the active form in inducing morphological transformation, while V(IV) is not transforming *(Sabbioni E. et al., 1991a)*. In the present work the carcinogenic potential of sodium metavanadate was showed already at concentration of 1 μM. Furthermore, vanadocene ($(C_5H_5)_2VCl_2$), an organic form of vanadium, was compared to the inorganic form for the cytotoxic response showing a slightly stronger cytotoxicity for the organic form (Table 13.III). Nevertheless, recent experiments have showed no carcinogenic potential for vanadocene unlike $NaVO_3 \cdot H_2O$ *(personal communication)*.

A further investigation performed at the end of morphological neoplastic transformation experiments has concerned the cloning of type III foci identified in living cell cultures previously treated with $NaAsO_2$, $CdCl_2 \cdot 2H_2O$, $(NH_4)_2PtCl_6$, $PtCl_2$, $PtCl_4$, carbo-Pt and *cis*-Pt (Sections 14.1 and 14.2). The purpose of this study concerns the evaluation of transformed features eventually displayed by selected Balb/3T3 clones (Section 14.4).

Since ECVAM is not working with laboratory animals, the part of the investigation using nude mice has been carried out at "Istituto Zooprofilattico Sperimentale dell'Emilia e della Lombardia" (IZS) of Brescia (Italy).

When clones were found able to form colonies in soft agar even after the second replicate, each of these clones was injected by subcutaneous route into two nude mice about 20-30 days old. Animals were observed weekly and in case of neo-formation they

were euthanised. Macroscopic and histopathologic investigations were carried out on their organs and tissues. Numerous clones derived from $NaAsO_2$, $CdCl_2 \cdot 2H_2O$, $(NH_4)_2PtCl_6$, $PtCl_2$, $PtCl_4$, carbo-Pt and *cis*-Pt produced colonies in soft agar (Photo 14.4). Interestingly, all clones showing this transformed feature presented morphology different from normal Balb/3T3 untreated cells during the amplification of the cultures. As illustrated in Photo 14.3, the typical fibroblastic-like appearance was lost and the cells appeared more spindle-shape or even epithelial-like. Experiments are in progress in order to verify if these changes are related to the acquisition of transformed properties (altered morphology).

However, at present only five clones derived from the treatment of 1 µM carbo-Pt and one from 5 µM $(NH_4)_2PtCl_6$ produced tumours in nude mice. The tumour was usually located in the area of injection. It was composed mostly of epithelial cells and showed high mitotic capacity. No metastases were observed, whereas modifications were identified in liver and spleen.

In vivo tests are currently in progress on further eight clones derived from $NaAsO_2$, $CdCl_2 \cdot 2H_2O$, $(NH_4)_2PtCl_6$, $PtCl_2$, and *cis*-Pt.

We can conclude that treatments of the Balb/3T3 cells using different metal compounds may induce modifications at genetic level, which result in morphological transformation for some cells within the population. This can be sometimes related to the acquisition of tumourigenic potential, as demonstrated by *in vivo* tests. If transforming activity of clones is identified by *in vitro* methods, the corresponding oncogenicity *in vivo* has to be confirmed. Consequently, it is very difficult to established quantitative correlation between transformation frequency *in vitro* and oncogenic activity *in vivo*. More specifically, the correlation existing among *in vitro* transformation assays, soft agar plating method and *in vivo* transformation should be considered very low. These findings highlight how it is important to understand if treatments of the Balb/3T3 cells using different metal compounds really can induce modifications at genetic level, which result in morphological neoplastic transformation and, sometimes, in the acquisition of tumourigenic potential *in vivo*.

In this context, the present study intends also to promote the prediction of carcinogenicity as well as to discriminate genotoxic/carcinogenic and non-genotoxic/carcinogenic metal compounds. This is indeed the principal aim of a

collaborative study on *in vitro* cell transformation assay for metal carcinogenesis performed by the Universitat Autònoma of Barcelona (Spain) in collaboration with the University of Pisa (Italy) and the "Instituto Zooprofilattico Sperimentale dell' Emilia e della Lombardia" (IZS) of Brescia (Italy). This project is focused on the use of the Balb/3T3 transformation assay particularly in combination with short term tests: a) the Human Lymphocyte *In Vitro* Micronucleus (MN) Assay coupled to Fluorescence *In Situ* Hybridisation (FISH) analysis to discriminate clastogenic/aneugenic potential of metal compounds; b) the Single Cell Gel Electrophoresis Assay (or Comet assay) performed on a human lymphoblastoid cell line (the TK6 cell line) in order to identify different mechanistic aspects related to the genotoxicity of the metals tested. These aspects should consider antigenotoxic, repair and oxidative damage induction.

Experiments are in progress to draw conclusions about the genotoxic potential of metal compounds, whose carcinogenic potential was showed in previously studies using the Balb/3T3 transformation assay. A first finding is the confirmation by MN test and Comet assay that the transforming $NaAsO_2$ and $Na_2HasO_4 \cdot 7H_2O$ display genotoxic potential and thus, they can be considered as genotoxic/carcinogenic metal compounds *(Guillamet E. et al., in preparation; Migliore L. et al., 1999; Migliore L. et al., 2002)*.

♦ **Studies on apoptosis.** The fourth step of the proposed work strategy concerned the investigation on the ability of $NaAsO_2$, $Na_2CrO_4 \cdot 4H_2O$, *cis*-Pt, carbo-Pt, $(NH_4)_2PtCl_6$, $PtCl_4$ and $PtCl_2$ to induce apoptosis in the Balb/3T3 cells (Sections 16.1-16.5). This represents a first attempt to give a mechanistic basis to cytotoxicity and carcinogenic potential of metal compounds observed in the present study.

Four approaches to identify different phases of the apoptotic process were applied: a) cytofluorimetric detection of early stages of apoptosis, as the rearrangement of the lipids in the plasma membrane (using the annexin V/PI assay); b) cytofluorimetric analysis of the induction of the caspase-3 activity, which is one of the 'effectors enzymes' of the cysteine-aspartate proteases family; c) nuclear fragmentation and d) chromatin condensation and apoptotic bodies formation related to the later stages of apoptosis, detected by TUNEL and Hoechst 33342/PI assays via confocal and fluorescence microscopy, respectively.

The findings obtained for $NaAsO_2$, $Na_2CrO_4 \cdot 4H_2O$, *cis*-Pt and carbo-Pt show that these metal compounds induced apoptosis in the Balb/3T3 cell line as determined by the

four methods applied, in agreement with the literature data (Section 2.1).

In particular, $Na_2CrO_4 \cdot 4H_2O$ showed DNA fragmentation also at 300 μM after 12 hours, whereas annexin V/PI, caspase-3 activity and Hoechst 3342/PI assays confirmed induction of apoptosis only after 6-hour exposure (Section 16.5). Probably, this is due to a combination of factors (concentration, exposure time) defining a late stage of the apoptotic process for this treatment. Consequently, this situation can be revealed only by techniques that directly analyse the nucleus, such as the applied DNA fragmentation assay (Photo 16.8).

Among the inorganic Pt-compounds tested $PtCl_4$ induced apoptosis, although the annexin V/PI assay gave negative results. However, annexin identifies rearrangements in the plasma membrane at very early stages of apoptosis. Thus, it is plausible to suppose that this assay is often not able to confirm apoptotic data in comparison to other methods detecting markers at nuclear level. Moreover, caspase-3 substrate recently was found related to nuclear changes associated with apoptosis *(Liu X. et al., 1997)*. Actually, caspases act not only on activation of other caspases (or pro-caspases) but also on components of the cytoskeleton (e.g. actin) and on nuclear proteins. Studies demonstrate that caspase-3 can cleave and activate the 45 kDa subunit of a protein, named DNA fragmentation factor, which leads to induction of nuclease activity, nuclear condensation and degradation of DNA into nucleosomal fragments *(Liu X. et al., 1997)*. This could explain the induction of apoptosis detected for $PtCl_4$ performing analysis on caspase-3 activity, DNA fragmentation and chromatin condensation (Section 16.5).

On the contrary, $(NH_4)_2PtCl_6$ and $PtCl_2$ can be considered non-inducers of apoptosis in the Balb/3T3 cell line, in spite of induction of caspase-3 activity at particular concentrations and exposure times (Section 16.5).

Table III summaries the findings achieved in this study concerning apoptosis induced by metal compounds in the Balb/3T3 cells.

Table III: Apoptosis determined by different methods in Balb/3T3 cells exposed to metal compounds

Metal compound	Concentration / Exposure	Apoptosis			
		AnnexinV/PI	Caspase-3 activity	DNA fragmentation	Hoechst/PI
$NaAsO_2$	100-175µM / 6h	+	+	+	+
$Na_2CrO_4 \cdot 4H_2O$	250-350µM / 6h	+	+	+ [a]	+
cis-Pt	85-150µM / 12h	+	+	+	+
carbo-Pt	1250-1750µM / 12h	+	+	+	+
$PtCl_4$	100-150µM / 12h	-	+	+	+
$(NH_4)_2PtCl_6$	100µM / 6h 75µM / 12h	-	+	-	-
$PtCl_2$	75µM / 6-12h	-	+	-	-

a: found positive also at 300 µM after 12-hour exposure.

A general comment concerns the failures to determine apoptosis and the difficulties in interpreting data as arisen from the present study, particularly for $(NH_4)_2PtCl_6$ and $PtCl_2$ and, to a less extent, also for $Na_2CrO_4 \cdot 4H_2O$ and $PtCl_4$. We judge these problems as foreseeable, particularly when the endpoint of each method applied for the analysis refers to a different step of apoptosis. In fact, in studying apoptosis the following remarks must be taken into account *(Darzynkiewicz Z. et al., 2001)*: *i*) the entire apoptotic process, from the initiation to the total disintegration of the cell, is of short and variable duration, which is different in different cell types and tissues, as well as *in vivo* and *in vitro*; *ii*) some inducers may slow down or accelerate the apoptotic process (e.g. protease inhibitors delay nuclear fragmentation and prolong the process of apoptosis; *iii*) limitations and possible pitfalls are intrinsic of every approach used to detect apoptosis. For example, the permeability and asymmetry of plasma membrane phospholipids (accessibility of phosphatidylserine) may change as a result of prolonged

treatment with proteolytic enzymes (trypsinisation), mechanical damage (e.g. cell removal from flasks, repeated centrifugations), or treatment with different drugs. Then, plasma permeability may vary depending on the cell type and on many factors, unrelated to apoptosis or necrosis *(Darzynkiewicz Z. et al., 2001)*.

Thus, many cautions must be taken in interpretating the data on apoptosis. This latter is a kinetic event and the time window during which individual apoptotic cells demonstrate their characteristic features (markers) that allow then to be recognisable varies depending on: *i*) the method used; *ii*) the cell type; and/or *iii*) the nature of the inducer of apoptosis.

♦ **Cytotoxicity studies on HaCaT cell line.** The cytotoxicity induced in the HaCaT cells after exposure to selected metal compounds ($(NH_4)_2PtCl_6$, $(NH_4)_2PdCl_6$, $(NH_4)_3RhCl_6$, $NaAsO_2$, $Na_2HAsO_4 \cdot 7H_2O$, and $(CH_3)_3AsCH_2COO^-$) (Section 17) is discussed with reference to the corresponding data obtained in the Balb/3T3 cells.

Both cell lines, when exposed to different inorganic and organoarsenic compounds, confirmed that only the inorganic trivalent and, to a less extent, pentavalent As-species were cytotoxic in a dose-dependent fashion (Figures 13.2 and 17.1). The corresponding IC_{50} values derived for As(III) and As(V) were also similar (1.5 μM and 5 μM, Balb/3T3 cells; 0.5 μM and 5 μM, HaCaT cells). As for the Balb/3T3 cells, unlike inorganic $NaAsO_2$ and $Na_2AsO_4 \cdot 4H_2O$, As-betaine did not induce significant cytotoxicity in the HaCaT cell line to concentrations up to 1000 μM (Figure 17.1).

The experiments on the cytotoxic effect induced in the HaCaT cells exposed to $(NH_4)_2PtCl_6$, $(NH_4)_2PdCl_6$, and $(NH_4)_3RhCl_6$ (Table 17.I) showed the same ranking of toxicity observed for the Balb/3T3 cells:

$$Pt(IV) > Rh(III) > Pd(IV).$$

This confirms once more that Pt(IV) is a very toxic chemical form (IC_{50} value of 3.7 μM and 30 μM for the Balb/3T3 and the HaCaT cells, respectively). The IC_{50} values for Rh(III) (160 μM, Balb/3T3; 100 μM, HaCaT) and Pd(IV) (230 μM, Balb/3T3; higher than 100 μM, HaCaT) were similar in both cells. Furthermore, this trend of cytotoxicity identified in the HaCaT cell line also confirms previously data obtained using the Neutral Red Uptake (NRU) assay *(Cocco B., 1999)*.

♦ **Studies on Syrian Hamster Embryo cell line.** Syrian Hamster Embryo cell transformation assay using primary cells generally is considered as a valuable test for the study of the carcinogenic potential of chemicals *(LeBoeuf R.A. et al., 1990)*.

Investigations carried out in this work on the SHE cell line (Section 18) showed experimental complications affecting the performance of the assay. One of the main difficulties was the modified pH value (pH = 6.7) of culture medium as proposed by LeBoeuf (pH = 6.7 obtained after incubation of culture medium at 10% CO_2 and 37°C in 90% humidified air) *(Kerckaert G.A. et al. 1996)*. We were unable to reproduce such experimental conditions. In fact, the initial value pH = 7.4, was unchanged over many days under the LeBoeuf conditions (data not showed). At this pH, however, the cell survival was about 10-fold higher compared to the acid pH (Table 18.II). Preliminary experiments at pH = 6.7 showed colony formation only after 72-hour exposure (but not after 24-hour exposure) with a mean of colonies/plate less than 15 (data not showed). Thus, the suggested modification of the assay at pH = 6.7 would not increase the sensitivity of the SHE assay (Section 1.3).

A recent review from OECD (Organisation for Economic Co-operation and Development) concerning *in vitro* transformation assays indicates that sensitivity, specificity and predictivity values are very similar between experiments carried out at pH = 6.7 and at pH = 7.35 *(personal communication)*. Thus, the value of the pH in the culture medium is not a determining factor for morphological transformation of the SHE cell line. It seems to be dependent on the culture medium conditions adopted in each laboratory.

In the present work another severe problem in the use of SHE cells has concerned the reproducibility of the assay. Since ECVAM is not allowed to use animals, it was not possible to sacrifice pregnant hamsters in order to prepare the feeder cells necessary for carrying out cytotoxicity and morphological transformation experiments. Consequently, these cells were obtained from an external supplier in frozen vials. We remind that the SHE assay needs two types of cells, target and feeder cells. The feeder cells are inactivated (X-irradiated) cells, which can no longer divide but can provide factors to support the growth of the target cells and improve their cloning efficiency (Section 6.1). It was impossible after thawing to reach an adequate number of feeder cells to seed in cytotoxicity experiments (receiving irradiated cells in culture was rejected due to frequent contaminations), probably because of an increased sensitivity of these cells

after irradiation. Thus a huge number of pregnant hamsters should be sacrificed to apply the SHE assay.

♦ ***In vitro*** **cellular biotransformation and interaction of metal compounds in culture medium and cellular lysate.** The availability of mechanistically based *in vitro* toxicity testing is a key aspect to improving the scientific basis of toxicity testing and non-animal testing strategies *(Balls M., 1998)*. This approach is taken into account by the IMETOX project that foresees the determination of the behaviour of metal compounds in cell culture medium as well as the identification of metabolic patterns of the metal incorporated into cells *(Sabbioni E. et al., 1999)*. The biochemical mechanisms can be elucidated by a combination of pharmacokinetic and metabolic studies (biotransformation, identification of metallobiocomplexes and determination of the oxidation state and their changes in the cell) *(Sabbioni E. et al., 1985)*. In this context, the delineation of metabolic pathways and kinetic patterns of metals in culture medium and cells is a key point in understanding the factors that determine toxicological responses and in interpreting the corresponding cytotoxicity.

The measurements of metal bioavailability and uptake are the first steps that must be evaluated in the development of mechanistically based *in vitro* assays *(Sabbioni E. and Balls, M., 1995)*. However, their evaluation poses serious technical problems. In fact, cell cultures are grown in a chemically defined medium, generally with the addition of foetal calf serum. The medium is a complex mixture (buffered saline solution containing amino acids, carbohydrates, vitamins, minerals, cofactors and sometimes pH indicator, separating buffered systems, and some non-essential amino acids, which may be used by particular cell type). This complex nature of culture medium may strongly influence cellular uptake and disposition of the assayed metal, e.g. by changing the simple original ionic form of this metal that represents the most common form in which metal compounds are generally tested. This is due to the possibility of metal ion to bind the numerous organic ligands, such as biochemicals and serum components of the medium, which can lead to the formation of metal-chelates and to oxido-reduction reactions that change the original oxidation state of the metal.

In addition, there is evidence that cells can biotransform the incorporated metal species, leading to metal metabolites of great toxicological significance. Typical examples are: *i*) the correlation between Cr(III)-induced DNA single-strand breaks and

the level of Cr(III) generated in Chinese Hamster V79 cells upon the metabolic reduction of Cr(VI) by glutathione or H_2O_2 *(Aiyar J. et al., 1991)*; *ii*) the reduction of toxic and morphological transforming V(V)-species into the less toxic and non-transforming V(IV) form by the Balb/3T3 cells, which represents the mechanism of detoxification for the toxic pentavanadate *(Sabbioni E. et al., 1993)*. Thus, the study of the chemical form of a metal that enters the cell (e.g. possible metabolic-mediated oxido-reduction processes) may provide information about the capability of the cell to detoxicate toxic metal species or to generate reactive toxic intermediates. Moreover, the analysis of metal species in culture medium at the end of the assay may give insights into the eventual presence of 'metabolised' metal catabolites as released by cells.

In this context, the application of the High Field Nuclear Magnetic Resonance (NMR) spectroscopy to our study shows the great potential of this technique in mechanistically based *in vitro* toxicity testing. For 32 metal compounds similar results were obtained from all different culture media tested in absence of cells, with and without serum (Table 19.I). This is a fundamental aspect to be considered in evaluating stability and reproducibility of the experimental conditions under which one or more cell systems are tested.

Another interesting finding concerns the loss of NMR signals in the case of Pt-compounds related to His molecule. The loss of this signal in absence of cells (Figure 19.2) and its persistence in presence of cells (Figure 19.3) suggests that different conditions can influence the pathways of a metal by involving different chemical and biological processes, such as kinetic transport into or uptake by the cells.

With regard to the identification of metal species in incubation medium at the end of the assay after cell removal, interesting data are reported in Figures 19.4 and 19.5, which are related to the NMR analysis on cellular lysates. These analysis detected the loss of a specific signal common to $NaAsO_2$, $Na_2CrO_4{\cdot}4H_2O$ and $(NH_4)_2PtCl_6$, but not to $NaVO_3{\cdot}H_2O$ (Figure 19.4). The findings that the original signal of the control consists of two overlapped signals and that the peak still visible after treatment corresponds to the reduced glutathione molecule (Figure 19.5) are useful information for the study still in progress to identify the second peak and consequently the pathway, in which $NaAsO_2$, $Na_2CrO_4{\cdot}4H_2O$ and $(NH_4)_2PtCl_6$ seem to be involved. Recent experiments suggest that this signal may correspond to an adenosilic derivate.

CONCLUSIONS

VS

OBJECTIVES OF THE PRESENT STUDY

♦ **Check of chemical purity.** The determination of the degree of elemental purity of metal compounds to be tested for cytotoxicity and carcinogenic potential by *in vitro* systems suggests a "low risk" of artefacts in relation to the biological response.

♦ **Standardisation and optimisation of Balb/3T3 cell transformation assay.** The achievements reached make the Balb/3T3 assay potentially available for future prevalidation/validation studies.

♦ **Determination of cytotoxicity and carcinogenic potential of metal compounds by Balb/3T3 assay.** The establishment of a database concerning cytotoxicity and morphological neoplastic transformation induced by metal compounds in the Balb/3T3 assay complements very well the corresponding existing database of organic compounds. The achievements about the carcinogenic potential of metal compounds are consistent and in good agreement with the corresponding *in vivo* situation. This assay is also a valuable *in vitro* model regarding the aspect of metal speciation, which is a key factor in determining metal toxicity, and for testing chemical mixtures.

♦ **Studies on apoptosis in the Balb/3T3 cell line.** New metal compounds have been identified and other confirmed as apoptotic agents. However, the studies carried out indicate that more than one assay should be used in order to establish unequivocally the induction of apoptosis by a metal compound. This is due to the complexity of the problem and the difficulties to interpret the experimental data. The findings on the apoptotic response induced by metal compounds suggest the hypothesis of an existing relationship among apoptotic, genotoxic and carcinogenic processes.

♦ **Cytotoxicity studies on HaCaT cell line.** The results achieved on cytotoxicity of selected metal compounds and the comparison with the corresponding data in Balb/3T3 cells not only suggest HaCaT cells as a reliable *in vitro* model for screening of cytotoxicity but also encourage to explore further the HaCaT cell line as *in*

vitro system of human origin for testing the carcinogenic potential of metal compounds.

♦ **Syrian Hamster Embryo (SHE) cell line.** The results highlight experimental difficulties of this *in vitro* system as cell transformation assay. However, we must take into account that this system is complementary to the Balb/3T3 assay because embryo cells (SHE) and established cell lines (Balb/3T3) are supposed to represent, respectively, early and later stages of the carcinogenesis process *(LeBoeuf R.A. et al., 1996)*. Thus, the coexistence of both these *in vitro* transformation assays may represent the best approach to predict *in vivo* carcinogenesis.

♦ **Cellular uptake, intracellular repartition and speciation in culture medium and cellular lysate.** The results obtained applying unique and peculiar analytical techniques like the use of radiotracers, ICP-MS and NMR have made possible to get interesting metabolic data on uptake and intracellular repartition of metals incorporated into cells. This is an aspect generally neglected in *in vitro* studies although it is a key point for mechanistic interpretation of cytotoxicity and transforming activity induced by chemicals.

Publications related to the present work

Mazzotti, F., Sabbioni, E., Ghiani, M., Cocco, B., Ceccatelli, R. and Fortaner, S. (2001). *In vitro* assessment of cytotoxicity and carcinogenic potential of chemicals: evaluation of the cytotoxicity induced by 58 metal compounds in the Balb/3T3 cell line. *ATLA* 29, 601-611.

Mazzotti, F., Sabbioni, E., Ponti, J., Ghiani, M., Fortaner, S. and Rossi. G.L. (2002). *In vitro* setting of dose-effect relationships of 32 metal compounds in the Balb/3T3 cell line, as a basis for predicting their carcinogenic potential. *ATLA* 30, 209-217.

Note on the nomenclature of figures, tables and photos

For ease identification of figures, tables and photos, a custom nomenclature was adopted. This is in direct relation to the graphical issues with the corresponding section.
Examples:
Figure 7.2 means the second figure included in section 7.
Table 5.IV means the fourth table that can be found in section 5.
Photo 4.3 means the third photo inserted in section 4.

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