RESULTS

12 Standardisation and Optimisation of Balb/3T3 Cell Line

This section concerns the results of the experiments carried out to select the best reagents and cells (culture medium, serum, source of Balb/3T3 cell line) and to examine other parameters (pH, solubility and chemical purity of metal solutions) that could influence the CFE response in untreated control or metal-exposed cells. In such experiments, $(NH_4)_2PtCl_6$ was used as positive control of cytotoxicity, as established by preliminary experiments (data not shown). The experimental protocol used in these experiments was that described in the cytotoxicity screening study (Figure 4.5 and Section 13.1) with modifications of the individual parameters under investigation.

12.1 Culture Medium and Serum

Table 12.I shows the effects of different culture media and sera on CFE in untreated or exposed to 10 μM of $(NH_4)_2PtCl_6$ Balb/3T3 cells.

DMEM from EURO CLONE and FCIII serum from HY CLONE (CFE 2) was the best medium/serum combination in terms of the yield of CFE (100 colonies) in relation to the number of cells (200 cells/dish), as foreseen by DiPaolo's protocol *(DiPaolo J.A. et al., 1972)*.

Table 12.I: Effect of culture medium and serum on cytotoxicity (CFE)

$(NH_4)_2PtCl_6$ (µM)	CFE 1 [a]			CFE 2 [a]			CFE 3 [a]		
	Colonies		% ± SEM [b]	Colonies		% ± SEM [b]	Colonies		% ± SEM [b]
Control	151	151	100	109	147	100	22	28	100
	150	150		144	134		25	51	
10	1	0	0.9 ± 0.7	0	1	0.6 ± 0.4	0	0	0.0
	3	2		1	2		0	0	

a: CFE 1: DMEM from GIBCO + FBS from GIBCO; CFE 2: DMEM from CELBIO + FCIII from CELBIO; CFE 3: DMEM from CELBIO + FBS from CELBIO.

b: 4 dishes/control, 4 dishes/treated cells. Average of 3 experiments: % of control ± SEM.

12.2 Solutions, Purity and Solubility of Metal Compounds

In order to avoid experimental artefacts due to possible metal impurities of metal solutions giving positive response in the transformation assay these latter were characterised for the elemental content by ICP-MS (Section 7.1) Table 12.II shows the results of the analysis of $(NH_4)_2PtCl_6$ solution (10^{-2} M).

Table 12.II: Determination of trace elements in $(NH_4)_2PtCl_6$ solution used in the determination of cytotoxicity and carcinogenic potential of the Pt-salt

Element	Concentration (µg/l)	Estimate of the element present as impurity in culture medium (M)
Al	1.3	5×10^{-8}
Au	0.03	2×10^{-10}
Ba	0.1	9×10^{-10}
Bi	0.003	1×10^{-11}
Cd	0.0007	6×10^{-12}
Ce	0.001	7×10^{-12}
Co	0.05	8×10^{-10}
Cs	0.00007	5×10^{-13}
Cu	0.2	2×10^{-9}
Fe	0.8	1×10^{-8}
Ir	0.006	3×10^{-11}
La	0.0009	6×10^{-12}
Nb	0.001	1×10^{-11}
Pb	0.4	2×10^{-9}
Pd	0.0003	3×10^{-12}
Rh	0.0002	2×10^{-12}
Se	0.3	3×10^{-9}
Sn	0.006	5×10^{-11}
Sr	0.1	$2x\ 10^{-9}$
Th	0.0007	3×10^{-12}
U	0.00004	3×10^{-12}
W	0.04	2×10^{-10}

The results show that Ag, As, Be, Ga, Hg, Mn, Mo, Sb, Te, Tl, Zn, and Zr, were below the experimental detection limit (less than 0.001 µg/l; results not shown). In any case, the estimated concentration of the elements in culture medium after addition of the Pt-salt was less than 10^{-8} M.

Since it is known that Pd-salts are not easily soluble, the solubility of the two species tested ($(NH_4)_2PdCl_4$ and $(NH_4)_2PdCl_6$) with oxidation state +2 and +4, respectively, was checked by ICP-MS at the nominal concentration of 50 µM in culture medium with or without serum (Section 7.2).

Table 12.III shows that both salts in culture medium with or without serum have mean Pd concentrations ranging from 48.5 µM to 51.0 µM compared to the corresponding theoretical value of 50 µM.

This confirms that Pd was uniformly distributed in culture medium under our experimental conditions.

Table 12.III: Check of the solubility of Pd in DMEM

Culture medium	Metal compound	Pd concentration (µM ± SEM) [a]
DMEM without serum	$(NH_4)_2PdCl_6$	50.5 ± 2.3
	$(NH_4)_2PdCl_4$	51.0 ± 1.2
DMEM with serum	$(NH_4)_2PdCl_6$	48.5 ± 2.0
	$(NH_4)_2PdCl_4$	49.0 ± 1.8

a: Average of 3 experiments. Experimental concentration ± SEM.

12.3 Source of Balb/3T3 Cell Line

Table 12.IV shows the influence of two different sources of Balb/3T3 cells (Section 4) on colony formation in untreated cells (control) or cells exposed to concentrations of $(NH_4)_2PtCl_6$, ranging from 0.5 μM to 3 μM.

Table 12.IV: Effect of the source of cell line on CFE [a]

Concentration (μM)	**Source 1** [b] **(nº colonies)**		**Source 2** [b] **(nº colonies)**	
	Mean	**Range**	**Mean**	**Range**
Control	180	172 – 195	107	97 – 112
0.5	126	120 – 131	91	80 – 101
1	126	118 – 141	84	76 – 88
3	80	63 – 94	71	61 – 79

a: 200 cells/dish; 6 dishes/control and treated cells. Average of 3 experiments.

b: source 1: ECVAM. Source 2: Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Brescia, Italy.

Taking into account that in the Balb/3T3 assay the optimum number of plated cells as control (200 cells/dish) produces 100 colonies *(DiPaolo J.A. et al., 1972)*, the source 2 was proved as the most reliable cell line to be assayed.

12.4 Effect of pH of Culture Medium on Cell Growth

Table 12.V shows the results related to the variation of pH of DMEM after 72-hour incubation of culture medium (with or without cells) with 1 μM of $(NH_4)_2PtCl_6$ compared to the corresponding values of pH at the beginning of the experiment. The same table shows also the effects of pH on the CFE values in the cells exposed to the Pt-salt.

At the end of the experiment the values of pH of free-cell culture medium and medium after incubation with cells ranged from 6.7 to 8.1 and 6.6 to 8.2, respectively. The highest CFE value is obtained at initial pH = 8.

Table 12.V: Variation of pH of culture medium at the beginning of the experiment (t=0) and 72 hours after incubation with 1 μM of $(NH_4)_2PtCl_6$ with or without cells

Beginning of the experiment [b] (t = 0)	pH values		
	End of the experiment (t = 72 h)		
	Without cells	With cells	CFE [a]
7.7 [c]	8.1	7.9	100 [d]
5.5	6.7	6.6	24.1 ± 2.2
6.0	7.5	7.6	76.8 ± 1.7
7.0	7.7	7.9	78.1 ± 4.7
8.0	8.0	8.1	82.3 ± 5.7
9.0	8.1	8.2	34.8 ± 4.2

a: average of 3 experiments. % of control ± SEM. 6 dishes/control and treated cells.

b: pH values obtained by adding HCl or NH_4OH.

c: original value of the medium without any adjustment of pH.

d: control without $(NH_4)_2PtCl_6$.

12.5 Effect of Thawing on Cell Growth

Table 12.VI shows the effects of the two different protocols for cell thawing (Section 4.1, Figure 4.3) tested on cell growth. Protocol 1 gave the best results, producing a number of cells/dish 3-fold higher than protocol 2.

Table 12.VI: Effect of thawing

	Cell growth [a] (nº cells/dish)
Protocol 1	3.1×10^6
Protocol 2	9.3×10^5

a: growing time: 5 days . Change of medium: twice.
Culture dishes: 100 x 20mm. Average of 3 experiments. (SEM < 15%).

12.6 NRU and CFE Assays

Table 12.VII shows a comparison between two basic assays to measure general cytotoxicity: NRU and CFE (Sections 4.3 and 4.2, respectively).

Both assays gave a dose-response curve. However, the percentage of cell survival at the same metal concentrations was obviously higher in the NRU assay than in the CFE assay, the protocol for which was optimised in this work.

Table 12.VII: Cytotoxicity induced in Balb/3T3 cells after 72-hour exposure to different concentrations of $(NH_4)_2PtCl_6$ as determined by NRU and CFE

Concentration (μM)	Cell survival (% of control ± SEM) [a]	
	NRU	CFE
Control	100	100
1	109.5 ± 0.08	80.4 ± 4.9
3	82.8 ± 0.03	60.0 ± 2.6
5	61.2 ± 0.09	37.3 ± 2.3
7	41.4 ± 0.01	22.6 ± 1.3
10	25.9 ± 0.02	9.2 ± 1.5
30	10.3 ± 0.01	-
50	10.3 ± 0.03	0
70	9.5 ± 0.0	0
100	6.0 ± 0.01	0

a: average of 3 experiments.

12.7 Reproducibility of CFE Assay

Table 12.VIII shows intralaboratory reproducibility of the CFE test as determined by four different operators on three different days after 72-hour after exposure to 5 μM $(NH_4)_2PtCl_6$. On a single day, mean variations of CFE among the four operators were 19%, 10.4% and 9.9% respectively, while the variations on the three different days were 19.9%, 16.8%, 26.7% and 28.1% for the individual operators.

These findings suggest that mean variations for the same operator are greater than those for different operators. This could be explained as differences in the experimental conditions that are more similar when the experiments were carried out on the same day than on different days.

Table 12.VIII: Reproducibility of CFE test

Operator	CFE [a] (% of control ± SEM) [b]		
	Day 1	Day 2	Day 3
1	100	100	100
	37.2 ± 3.7	35.7 ± 2.6	29.9 ± 4.8
2	100	100	100
	37.5 ± 3.7	35.0 ± 4.1	31.2 ± 4.5
3	100	100	100
	42.0 ± 4.8	39.0 ± 4.4	30.8 ± 0.9
4	100	100	100
	34.0 ± 5.2	39.1 ± 4.7	28.1 ± 1.2

a: 6 dishes/control and treated cells. Every day the operators used cells (source 2, see Table 12.IV) at the same passage.

b: average of 3 experiments.

13 Cytotoxicity of Metal Compounds in Balb/3T3 Cell Line

This section concerns the results of the first step of a working strategy concerning the determination of concurrent cytotoxicity and carcinogenic potential of metal compounds by the Balb/3T3 cell transformation assay. This working strategy involves: a screening of a large number of metal compounds at a fixed concentration to establish a ranking of cytotoxicity (Section 13.1); selection of metal species on the basis of the screening for setting the corresponding dose-response curves in order to calculate the 50% inhibition concentration values by a statistical treatment (Section 13.2) and derive the appropriate metal concentration for the transformation study.

A particular aspect underlined is the influence of metal speciation on the cytotoxic response (selected Pt- and As-compounds, Sections 13.3 and 13.4).

Finally, this section reports the results concerning cytotoxicity induced by combined mixtures (cases of hard metals and platinoids) (Section 13.5).

13.1 Screening Study

Table 13.I shows the cytotoxic effect induced by 65 metal compounds in Balb/3T3 cells after 72-hour exposure to 100 μM of each compound (three screening runs). The results of CFE (expressed as percentage of the control) were classified as three different groups, according to the degree of the cytotoxic effect:

- **group I**: cell survival higher than 80% [from 97.2% (H_3BrO_3) to 80.3% ($(NH_4)_2PdCl_6$)];
- **group II**: cell survival ranging from 30% to 80% [from 78.9% ($SnCl_2{\cdot}2H_2O$) to 32.3% ($(NH_4)_2PtCl_4$)];
- **group III**: cell survival less than 30% [from 28.0% ($CoSO_4{\cdot}7H_2O$) to complete growth inhibition ($AgNO_3$, $NaAsO_2$, $Na_2HAsO_4{\cdot}7H_2O$, $Bi(NO_3)_3{\cdot}5H_2O$, $CdCl_2{\cdot}2H_2O$, $CdMoO_4$, $Na_2CrO_4{\cdot}4H_2O$, $Ga(NO_3)_3{\cdot}6H_2O$, $HgCl_2$, CH_3HgCl, $MnSO_4{\cdot}5H_2O$, $(NH_4)_2PtCl_6$, Na_2TeO_3, $K_2TeO_3{\cdot}H_2O$, $(C_5H_5)_2VCl_2$, $NaVO_3{\cdot}H_2O$, $VOSO_4{\cdot}5H_2O$)].

Table 13.I: Cytotoxicity induced in Balb/3T3 cells after 72-hour exposure to 100 µM of 65 metal compounds classified as group I, II and III

CFE					
Group I		**Group II**		**Group III**	
Metal compound	**CFE (% of control ± SEM)**	**Metal compound**	**CFE (% of control ± SEM)**	**Metal compound**	**CFE (% of control ± SEM)**
Control	100	Control	100	Control	100
$Al(NO_3)_3 \cdot 9H_2O$	83.8 ± 0.4	$BeCl_2$	60.5 ± 5.8	$AgNO_3$	0.0
H_3BrO_3	97.2 ± 0.1	$KBrO_3$	44.2 ± 5.1	$NaAsO_2$	0.0
$(CH_3)_3AsCH_2COO^-$	93.5 ± 1.5	$NiSO_4 \cdot 7H_2O$	69.8 ± 0.8	$Na_2HAsO_4 \cdot 7H_2O$	0.0
$(CH_3)_2AsNaO_2 \cdot 3H_2O$	89.9 ± 0.5	$Pb(NO_3)_2$	61.0 ± 2.7	$AuCl_3$	2.5 ± 0.8
$CH_3AsO(OH)_2$	92.3 ± 0.1	$(NH_4)_2PtCl_4$	32.3 ± 3.6	$Bi(NO_3)_3 \cdot 5H_2O$	0.0
$Ba(NO_3)_2$	84.0 ± 1.4	$(NH_4)_3RhCl_6$	61.3 ± 4.2	$CdCl_2 \cdot 2H_2O$	0.0
KBr	97.0 ± 4.7	$SnCl_2 \cdot 2H_2O$	78.9 ± 0.7	$CdMoO_4$	0.0
$Ce(NO_3)_3 \cdot H_2O$	92.0 ± 7.4	$Na_2TeO_4 \cdot 2H_2O$	50.3 ± 4.7	$CoSO_4 \cdot 7H_2O$	28.0 ± 1.5
$CrCl_3 \cdot 6H_2O$	86.9 ± 4.5	$Th(NO_3)_4 \cdot 8H_2O$	73.2 ± 2.1	$Na_2CrO_4 \cdot 4H_2O$	0.0
CsCl	83.0± 2.0	$(C_5H_5)_2TiCl_2$	57.8 ± 3.2	$CuSO_4 \cdot 5H_2O$	15.9 ± 1.2
$GdCl_3 \cdot 6H_2O$	83.0 ± 2.0	$UO_2(NO_3)_2 \cdot 6H_2O$	74.0 ± 0.1	$Ga(NO_3)_3 \cdot 6H_2O$	0.0
GeO_2	88.8 ± 0.2	$Na_2WO_4 \cdot 2H_2O$	76.0 ± 0.1	$HgCl_2$	0.0
$HfCl_2O \cdot 8H_2O$	96.7 ± 2.4	$ZnSO_4 \cdot 7H_2O$	55.8 ± 0.3	CH_3HgCl	0.0
$InCl_3 \cdot 2\text{-}3H_2O$	83.5 ± 5.1			$(NH_4)_2IrCl_6$	5.5 ± 0.7
$La(NO_3)_3 \cdot 6H_2O$	80.8 ± 2.2			$(NH_4)_3IrCl_6 \cdot H_2O$	15.7 ± 2.0
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ [a]	80.8 ± 4.6			$MnSO_4 \cdot 5H_2O$	0.0
K_2MoO_4	83.4 ± 0.4			$(NH_4)_2PtCl_6$	0.0
$NbCl_5$	82.1 ± 5.1			Na_2TeO_3	0.0
$(NH_4)_2OsCl_6$	83.8 ± 2.7			$K_2TeO_3 \cdot H_2O$	0.0
$(NH_4)_2PdCl_4$	82.0 ± 4.8			$(C_5H_5)_2VCl_2$	0.0
$(NH_4)_2PdCl_6$	80.3 ± 2.4			$NaVO_3 \cdot H_2O$	0.0
RbCl	84.7 ± 0.5			$VOSO_4 \cdot 5H_2O$	0.0
NH_4ReO_4	82.8 ± 1.3				
$(NH_4)_2[Ru(H_2O)Cl_5]$	85.3 ± 1.8				
$K_4Sb_2O_7$ [a]	88.8 ± 4.9				
Na_2SeO_3	86.5 ± 0.3				
Na_2SeO_4	92.5 ± 1.4				
$Sr(NO_3)_2$	82.0 ± 3.0				
$(NH_4)_2[TiO(C_2O_4)_2] \cdot H_2O$	85.3 ± 4.4				
$Zr(NO_3)_4$	86.2 ± 2.0				

a: 400 µM as elemental Mo; 200 µM as elemental Sb.

13.2 Dose-effect Relationships of Metal Compounds

Figures 13.1 and 13.2 illustrate dose-effect curves concerning 35 metal compounds: 29 inorganic compounds (14 cationic and 15 anionic) and 6 organometallic species. They include 22 metal compounds from group III (strong or complete CFE inhibition), 5 of group II and 8 of group I.

The results are organised in order to show dose-response curves of 12 individual metal compounds (Figure 13.1), whose corresponding IC_{50} values ranged from 3 µM ($CdCl_2 \cdot 2H_2O$ and $CdMoO_4$) to 1573 µM ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) (Table 13.II). Moreover, the chemical forms of each metal compound tested (As, Br, Cr, Hg, Ir Pt, Te, Ti, and V) showed different slopes of the curve confirming different degree of cytotoxicity (Figure 13.2). The corresponding IC_{50} values ranged from 0.32 µM (CH_3HgCl) to 8380 µM (KBr) (Table 13.III). All metal compounds previously classified as group III (Table 13.I) depicted a dose-response fashion of cytotoxicity.

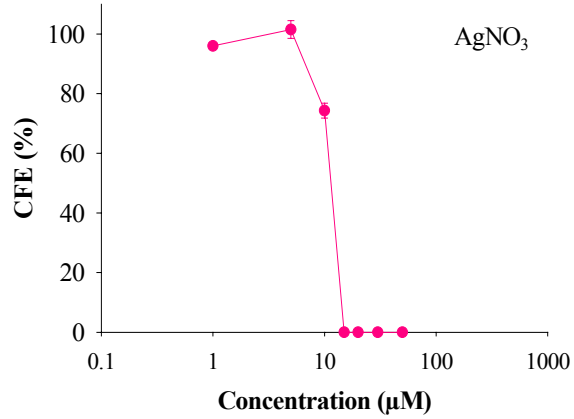
Table 13.II: Inhibitory concentration of 50% cell growth (IC_{50}) of metal compounds in Balb/3T3 cells

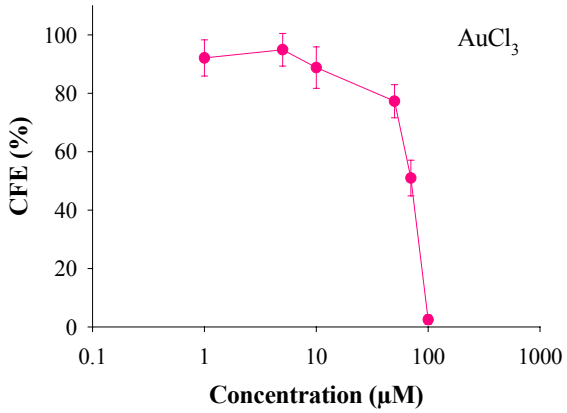
Metal compound	IC_{50} (µM) ± SEM
$AgNO_3$	11.2 ± 0.4
$AuCl_3$	72.5 ± 2.4
$Bi(NO_3)_3 \cdot 5H_2O$	23.6 ± 3.1
$CdCl_2 \cdot 2H_2O$	3.0 ± 0.01
$CdMoO_4$	3.0 ± 0.05
$CoSO_4 \cdot 7H_2O$	22.9 ± 0.4
$CuSO_4 \cdot 5H_2O$	71.5 ± 3.0
$Ga(NO_3)_3 \cdot 6H_2O$	60.7 ± 1.9
$MnSO_4 \cdot 5H_2O$	14.7 ± 2.4
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1573 ± 22 [a]
$(NH_4)_2PdCl_6$	230 ± 2.1
$(NH_4)_3RhCl_6$	160 ± 1.6

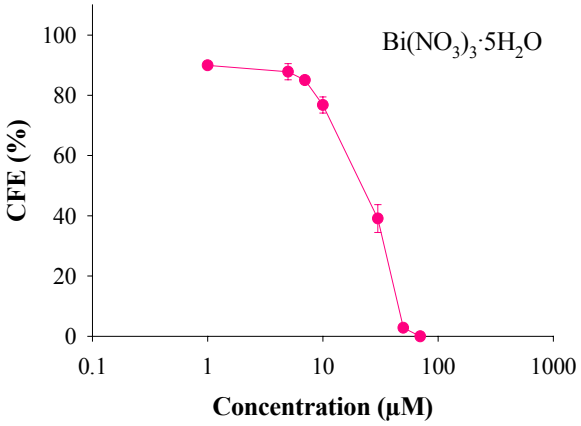
a: extrapoled value (USEPA, 1991) due to the low cytotoxicity at the tested concentrations (CFE inhibition not higher than 30% of the control).

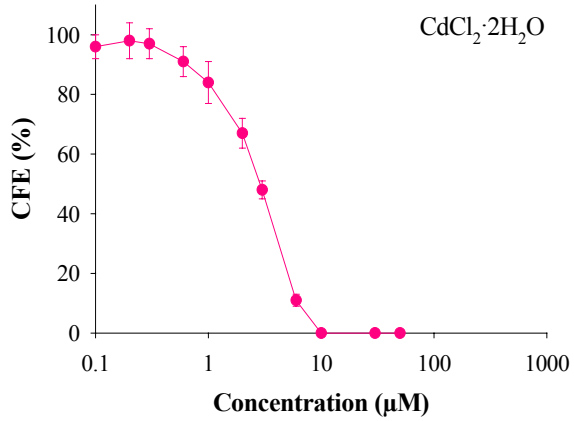
Figure 13.1: Dose-response curves of metal compounds in Balb/3T3 cells

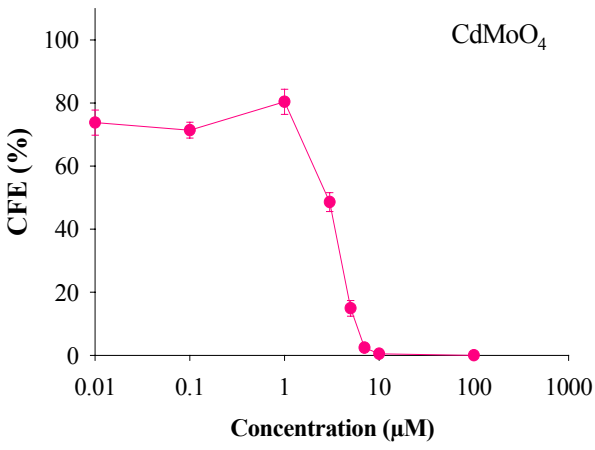
(for IC_{50} values see Table 13.II)

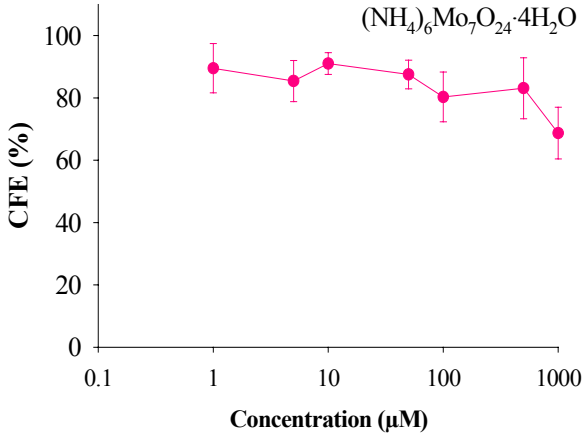


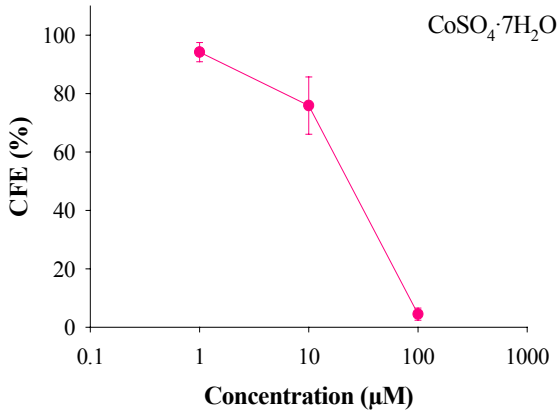


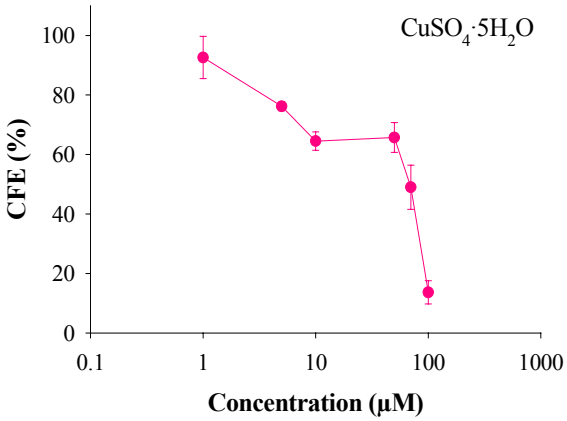


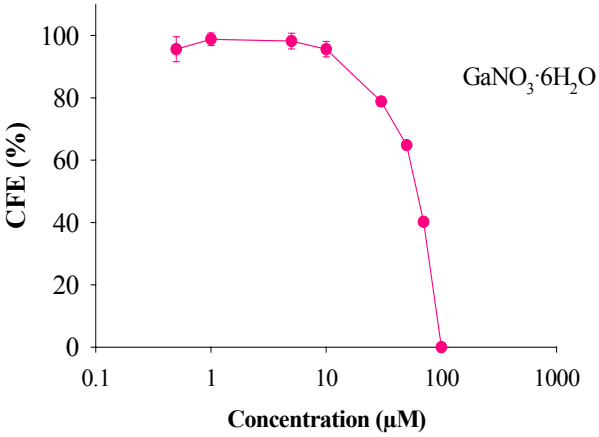


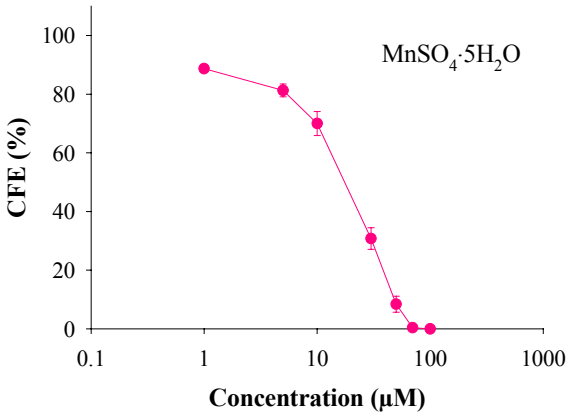


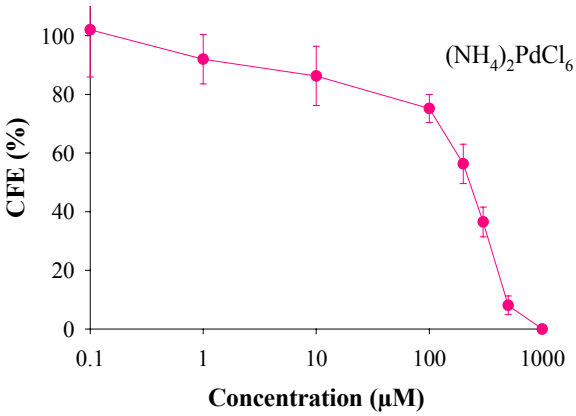



$CoSO_4 \cdot 7H_2O$
CFE (%)
Concentration (µM)


$CuSO_4 \cdot 5H_2O$
CFE (%)
Concentration (µM)


$GaNO_3 \cdot 6H_2O$
CFE (%)
Concentration (µM)


$MnSO_4 \cdot 5H_2O$
CFE (%)
Concentration (µM)


$(NH_4)_2PdCl_6$
CFE (%)
Concentration (µM)

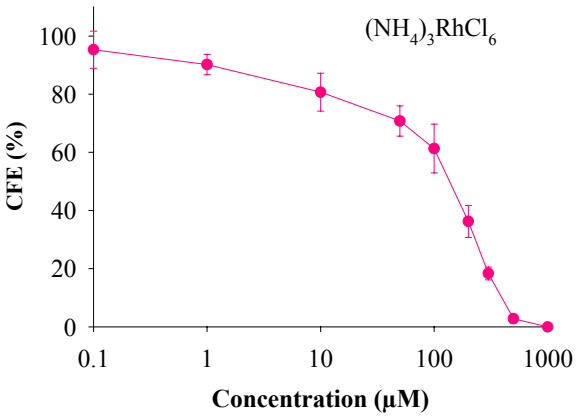
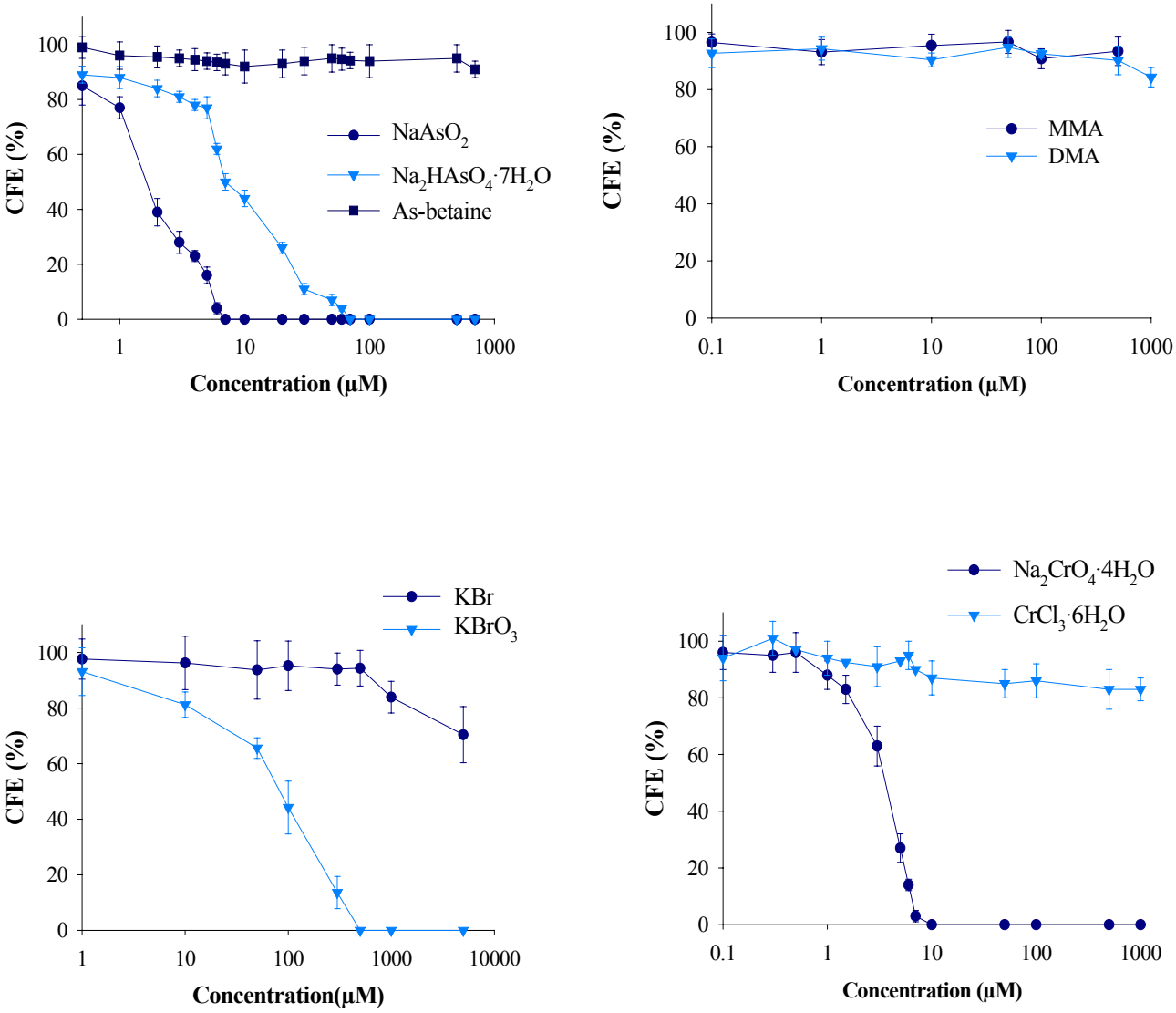

$(NH_4)_3RhCl_6$
CFE (%)
Concentration (µM)

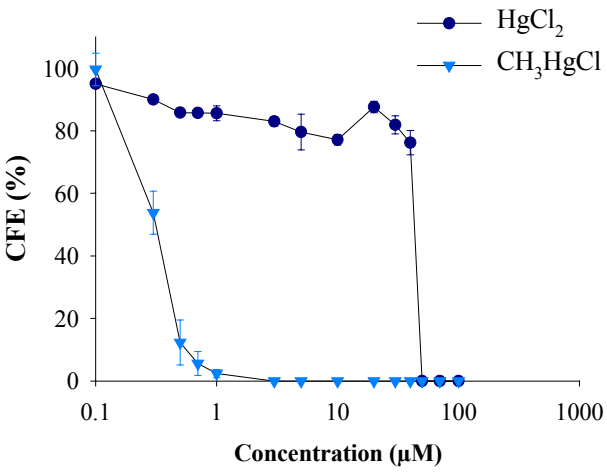
Table 13.III: Effects of metal speciation on IC_{50}

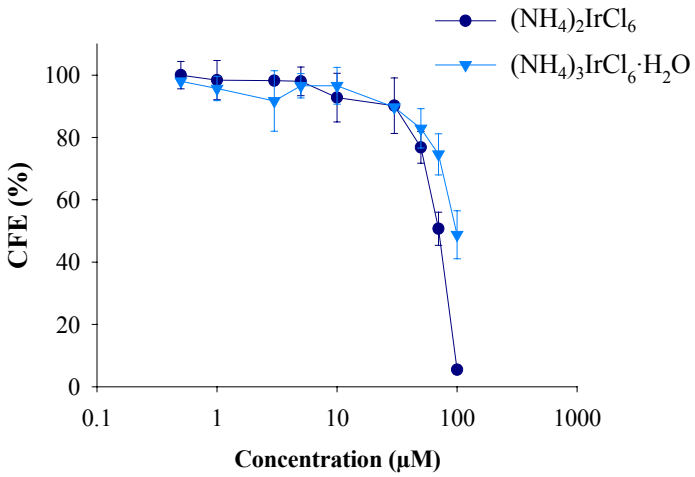
Metal compound	IC_{50} (µM) ± SEM
$NaAsO_2$	1.5 ± 0.01
$Na_2HAsO_4 \cdot 7H_2O$	5.0 ± 0.05
$(CH_3)_3AsCH_2COO^-$ (As-betaine)	3900 ± 32 [a]
$CH_3AsO(OH)_2$ (MMA)	3145 ± 28 [a]
$(CH_3)_2AsNaO_2 \cdot 3H_2O$ (DMA)	3158 ± 523 [a]
KBr	8380 ± 48 [a]
$KBrO_3$	80.3 ± 0.9
$CrCl_3 \cdot 6H_2O$	2841 ± 35 [a]
$Na_2CrO_4 \cdot 4H_2O$	3.6 ± 0.05
$HgCl_2$	43.1 ± 0.01
CH_3HgCl	0.32 ± 0.005
$(NH_4)_3IrCl_6 \cdot H_2O$	98.1 ± 0.3
$(NH_4)_2IrCl_6$	71.2 ± 0.1
$(NH_4)_2PtCl_4$	55.0 ± 0.2
$(NH_4)_2PtCl_6$	3.7 ± 0.05
Na_2TeO_3	3.8 ± 0.3
$K_2TeO_3 \cdot H_2O$	5.6 ± 0.05
$Na_2TeO_4 \cdot 2H_2O$	98.3 ± 4.7
$(NH_4)_2[TiO(C_2O_4)_2] \cdot H_2O$ (Ti-oxalate)	1598 ± 52 [a]
$(C_5H_5)_2TiCl_2$	117 ± 14.8
$VOSO_4 \cdot 5H_2O$	43.3 ± 0.5
$(C_5H_5)_2VCl_2$	2.3 ± 0.2
$NaVO_3 \cdot H_2O$	4.7 ± 0.05

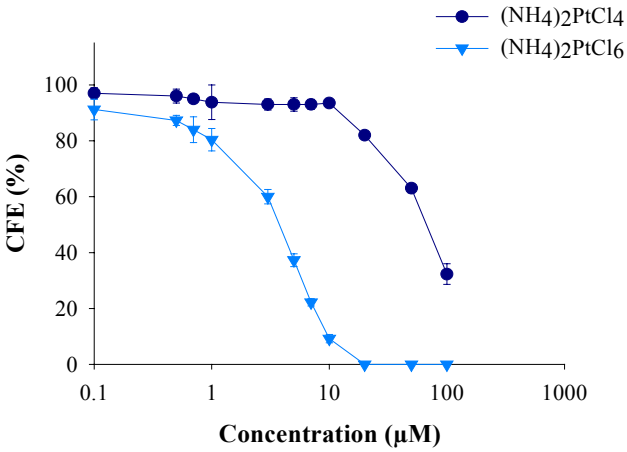
a: extrapoled value (USEPA, 1991) due to the low cytotoxicity at the tested concentrations (CFE inhibition not higher than 30% of the control)

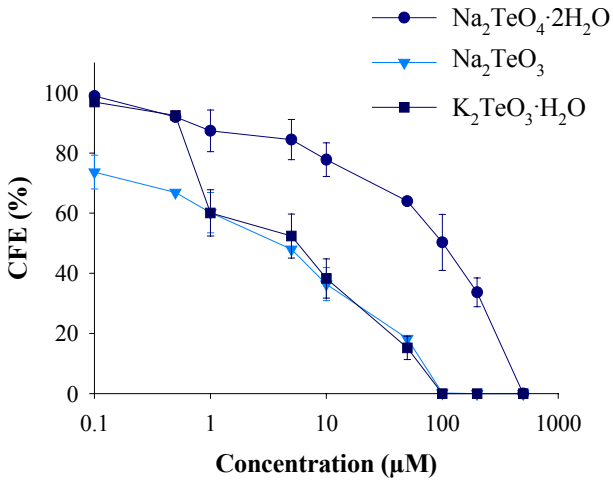
Figure 13.2: Dose-response curves of different chemical forms of individual metals in Balb/3T3 cells (for IC_{50} values see Table 13.III)

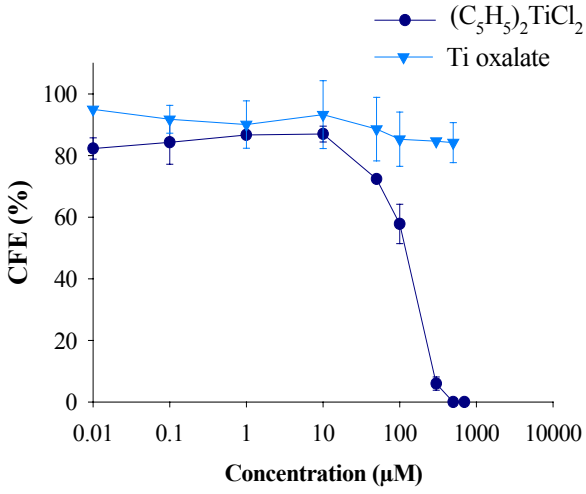


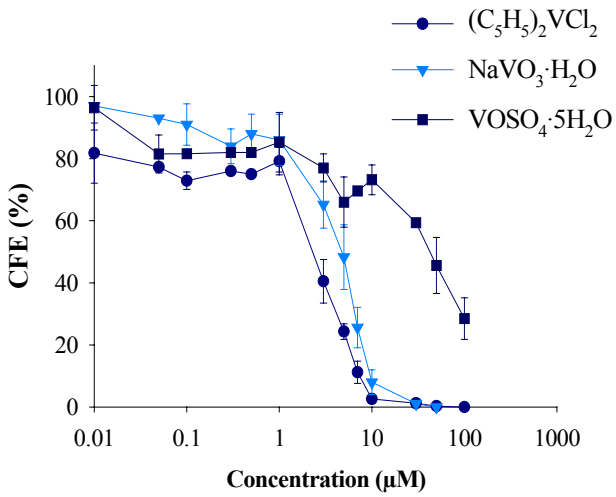

HgCl2
CH3HgCl
CFE (%)
Concentration (µM)


(NH4)2IrCl6
(NH4)3IrCl6·H2O
CFE (%)
Concentration (µM)


(NH4)2PtCl4
(NH4)2PtCl6
CFE (%)
Concentration (µM)


Na2TeO4·2H2O
Na2TeO3
K2TeO3·H2O
CFE (%)
Concentration (µM)


(C5H5)2TiCl2
Ti oxalate
CFE (%)
Concentration (µM)


(C5H5)2VCl2
NaVO3·H2O
VOSO4·5H2O
CFE (%)
Concentration (µM)

13.3 Cytotoxicity of Pt-compounds

The following Pt-compounds were selected in order to study the influence of metal speciation on the cytotoxic response in the Balb/3T3 cells: $(NH_4)_2PtCl_4$, $(NH_4)_2PtCl_6$, $PtCl_2$, $PtCl_4$, *cis*-Pt and carbo-Pt.

Figures 13.3-13.5 show the cytotoxicity induced by each pair of anionic (Figure 13.3) and cationic (Figure 13.4) inorganic species as well as inorganically complexed ions (*cis*-Pt) and organoplatinum compounds (carbo-Pt) (Figure 13.5) tested in a range of concentrations from 0.01 µM to 100 µM.

Figure 13.3: Dose-response curves of $(NH_4)_2PtCl_4$ and $(NH_4)_2PtCl_6$ in Balb/3T3 cells

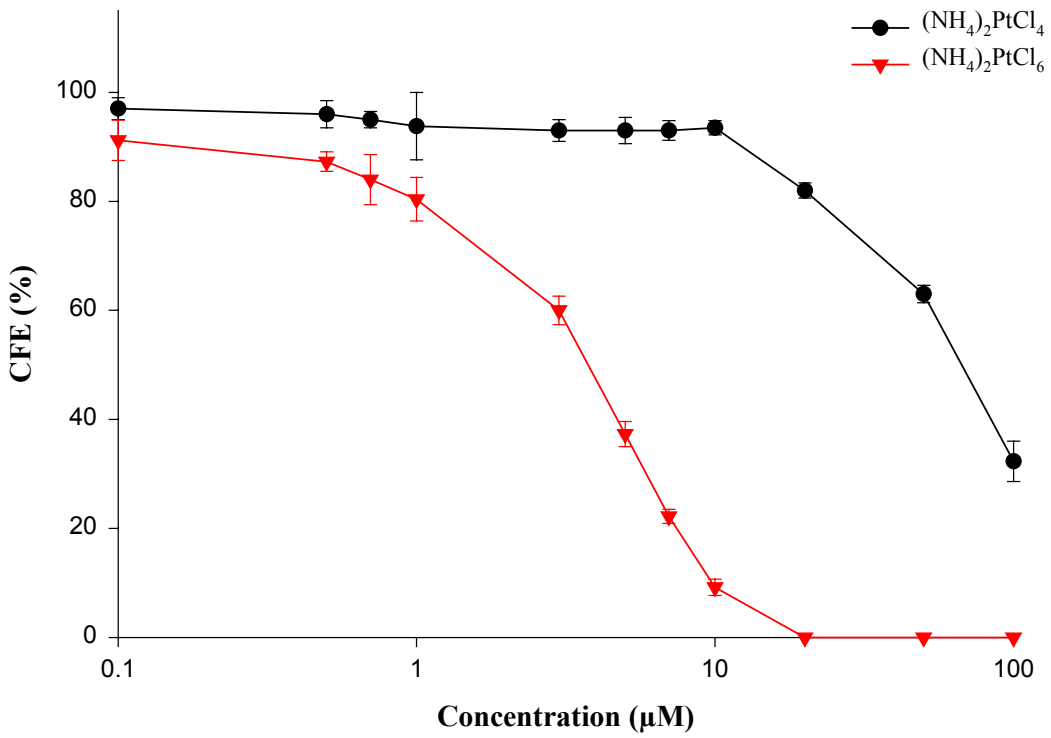


Figure 13.4: Dose-response curves of $PtCl_2$ and $PtCl_4$ in Balb/3T3 cells

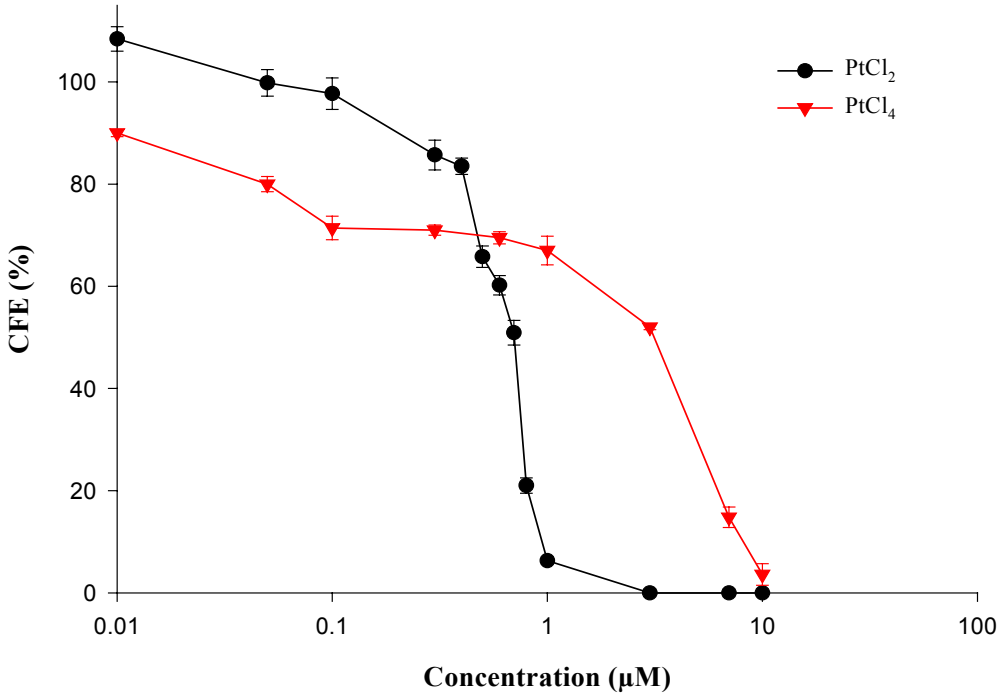
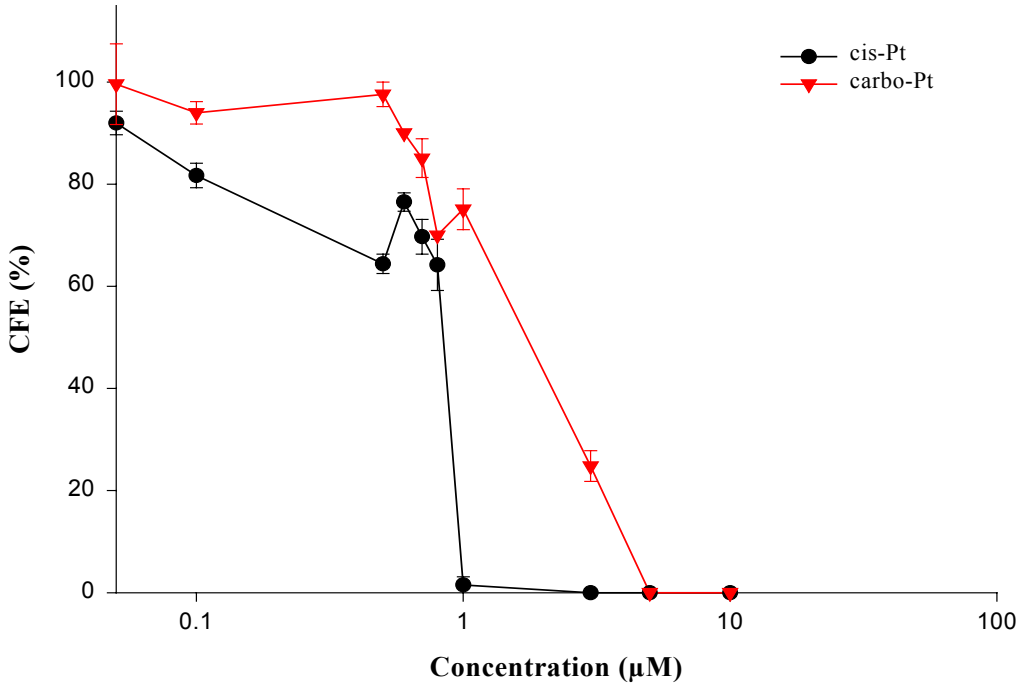


Figure 13.5: Dose-response curves of cis-Pt and carbo-Pt in Balb/3T3 cells



The oxidation state +4 as anionic form was more toxic than the corresponding salt with valence +2 (Figure 13.3). However, the situation appears to be the opposite for cationic and organoplatinum forms (Figures 13.4 and 13.5). In both cases Pt(II)-compounds showed a cytotoxic effect 4-fold stronger than Pt(IV)-compounds, both as inorganically complexed, organic and cationic forms (Table 13.IV).

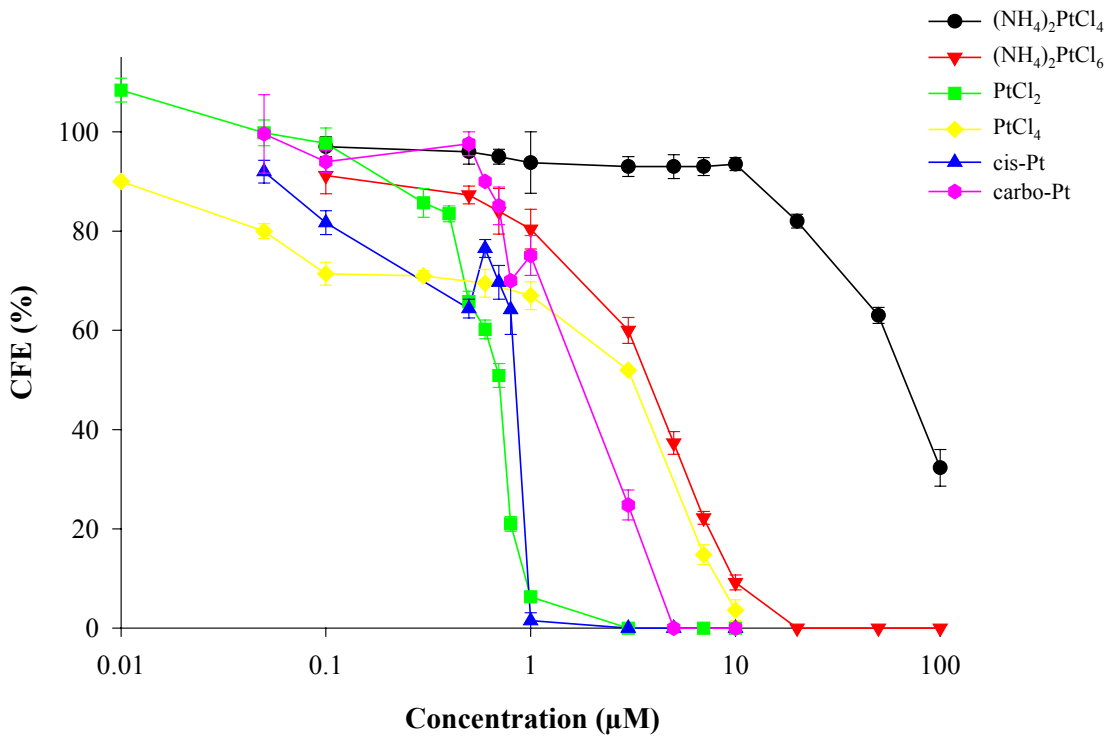
Table 13.IV: Inhibitory concentration (IC_{50}) of Pt-compounds in Balb/3T3 cells

Metal compound	IC_{50} (µM) ± SEM
$(NH_4)_2PtCl_4$	55.0 ± 0.2
$(NH_4)_2PtCl_6$	3.7 ± 0.05
$PtCl_2$	0.7 ± 2.0
$PtCl_4$	3.2 ± 1.0
cis-Pt	0.8 ± 3.3
carbo-Pt	1.8 ± 3.5

The overall situation is depicted in Figure 13.6 from which it is possible to derive the following ranking of cytotoxicity, which is also confirmed by the corresponding IC_{50} values reported in Table 13.IV:

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

Figure 13.6: Dose-response curves of the Pt-species in Balb/3T3 cells



Furthermore, Table 13.V shows the results of a screening study performed in the Balb/3T3 cells exposed to other four Pt-compounds at the concentration 100 μM.

Table 13.V: Screening of selected Pt-compounds in Balb/3T3 cells (72-hour exposure)

Metal compounds	CFE
$Na_2PtCl_6 \cdot 6H_2O$	0
$Na_2PtBr_6 \cdot 6H_2O$ [a]	0.3 ± 0.2
$Na_2Pt(OH)_6$	2.6 ± 0.4
$Na_2PtI_6 \cdot 6H_2O$ [a]	93.3 ± 2.4

a: internal control at 100 μM: KBr (94.1 ± 3.5) and NaI (91.8 ± 4.1).

It is to note that $Na_2PtCl_6 \cdot 6H_2O$, $Na_2PtBr_6 \cdot 6H_2O$ and $Na_2Pt(OH)_6$ showed a strong cytotoxic effect comparable to that detected for $(NH_4)_2PtCl_6$. Thus, it is possible to classify these Pt-compounds as group III of cytotoxicity (Section 13.1). On the other hand, no cytotoxic response was found for $Na_2PtI_6 \cdot 6H_2O$.

In the context of these experiments, in order to exclude any experimental artefacts due to the presence of I^- and Br^- ions, a further CFE study was carried out using KBr and NaI compounds at 100 μM. Both these species showed a cell viability of 94.1% ± 3.5 and 91.8% ± 4.1, respectively (see note *a*, Table 13.V). These data definitively excluded any effect of the I^- and Br^- ions in the cytotoxicity results of the corresponding Pt-salts complexed with these anions.

13.4 Cytotoxicity of As-compounds

The present section refers to the results of CFE in the Balb/3T3 cell line by exposure of cells to inorganic and organoarsenic species.

As already determined in previous studies (Tables 13.I and 13.III), inorganic but not organoarsenic compounds were classified as group III and showed a clear dose-response curve (Figure 13.2). However, Table 13.VI reports the particular case of the pentavalent As-compounds, $NaAsF_6$, $KAsF_6$ and $LiAsF_6$. At 100 µM none of such As-species significantly reduced cell survival, unlike the total growth inhibition induced by the As(V)-species previously tested: $Na_2HAsO_4 \cdot 7H_2O$ (Table 13.I).

Table 13.VI: Cytotoxicity of pentavalent inorganic As-compounds in Balb/3T3 cells exposed to 100µM (72-hour exposure)

Metal compound	CFE (% of control ± SEM)
$Na_2HAsO_4 \cdot 7H_2O$	0
$NaAsF_6$ [a]	87.6 ± 0.7
$KAsF_6$ [a]	91.5 ± 0.2
$LiAsF_6$ [a]	95.2 ± 0.4

a: internal control at 100 µM: LiF (88.9 ± 0.6).

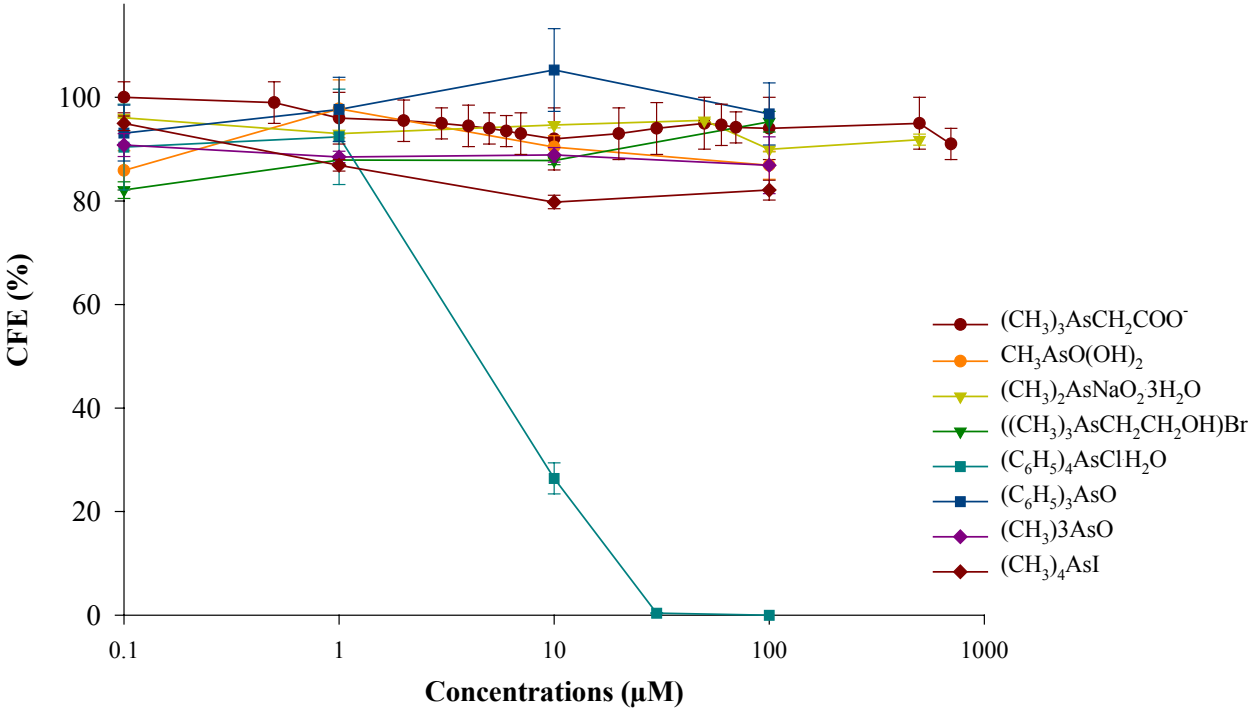
Furthermore, recent experiments (Table 13.VII) confirmed negligible cytotoxic effects for the following organoarsenic compounds: $(CH_3)_3AsCH_2COO^-$, $CH_3AsO(OH)_2$, $(CH_3)_2AsNaO_2 \cdot 3H_2O$, $((CH_3)_3AsCH_2CH_2OH)Br$, $(C_6H_5)_4AsCl \cdot H_2O$, $(C_6H_5)_3AsO$, $(CH_3)_3AsO$ and $(CH_3)_4AsI$. In the range of concentrations tested (from 0.1 μM to 100 μM) the decrease of cell survival was less than 20%, with the exception of $(C_6H_5)_4AsCl \cdot H_2O$ that induced a complete cell growth inhibition at 100 μM in a well-defined dose-response curve (IC_{50} = 4.8 μM) (Figure 13.7).

Table 13.VII: Cytotoxic effect of organoarsenic compounds induced in Balb/3T3 cells

Metal compound	CFE			
	Concentration (μM)			
	0.1	**1**	**10**	**100**
$(CH_3)_3AsCH_2COO^-$	100.0±3.0	96.0±5.0	92.0±6.0	94.0±6.0
$CH_3AsO(OH)_2$	85.9±3.8	97.8±5.6	90.4±2.2	86.9±2.7
$(CH_3)_2AsNaO_2 \cdot 3H_2O$	96.1±0.07	93.0±0.2	94.7±0.7	90.0±0.5
$((CH_3)_3AsCH_2CH_2OH)Br$ [a]	82.1±1.6	87.9±0.8	87.8±0.8	95.3±2.0
$(C_6H_5)_4AsCl \cdot H_2O$	90.4±8.3	92.4±9.2	26.4±12.5	0
$(C_6H_5)_3AsO$	93.1±5.4	97.7±6.2	105.3±8.0	96.8±6.0
$(CH_3)_3AsO$	90.8±2.2	88.5±1.1	88.9±1.4	86.9±5.5
$(CH_3)_4AsI$ [b]	95.0±1.4	86.9±1.1	79.8±1.3	82.1±1.9

a: internal control at 100 μM: KBr (94.1 ± 3.5) and NaI (91.8 ± 4.1).

Figure 13.7: Dose-response curves of organoarsenic compounds in Balb/3T3 cells



For all the experiments of section 13.4 any experimental artefact, due to the presence of F^-, Br^- and I^- ions, was definitively excluded because no cytotoxic response was found testing LiF (see note *a*, Table 13.VI), KBr and NaI (see note *a*, Table 13.VII).

13.5 Cytotoxicity of Combined Mixtures

Table 13.VIII shows the cytotoxic effect induced in the Balb/3T3 cells exposure to 10 μM of $(NH_4)_2PtCl_6$, plus $(NH_4)_2PdCl_6$, and $(NH_4)_3RhCl_6$, tested as combined mixture. In these experiments Pt(IV) (group III, Table 13.I) confirms to be the strongest cytotoxic compound compared to Pd(IV) and Rh(III) (groups I and II, respectively) and thus, it determines the cytotoxic response of the mixture.

Table 13.VIII: Cytotoxicity induced by a combined mixture of Pt-, Pd- and Rh-compounds in Balb/3T3 cells (72-hour exposure)

Metal compound	**CFE (% of control ± SEM)**
Control	100
Pt(IV)	8.4 ± 1.8
Pd(IV)	87.0 ± 4.8
Rh(III)	92.8 ± 5.3
Pt (IV) + Pd (IV) + Rh (III)	2.8 ± 0.8

Table 13.IX reports the results obtained in the Balb/3T3 cells exposed to 10 μM of individual metal compounds of the following mixtures:

- $CoCl_2 \cdot 6H_2O + K_2MoO_4 + (NH_4)_2[TiO(C_2O_4)_2] \cdot H_2O + Na_2WO_4 \cdot 2H_2O$;
- $CoCl_2 \cdot 6H_2O + K_2MoO_4 + (NH_4)_2[TiO(C_2O_4)_2] \cdot H_2O + Na_2WO_4 \cdot 2H_2O + NaVO_3 \cdot H_2O$.

The results showed no appreciable toxic effects of Co or Mo alone as well as when Co was tested in presence of Co, Mo, Ti and W. However, in presence of V(V) the cytotoxic effect of the mixture became very strong leading to a complete growth inhibition. This confirms the specific action of the V(V) (group III, Table 13.I) suggesting no synergistic effects with the other metal compounds tested.

Table 13.IX: Cytotoxicity induced by combined mixtures of Co-, Mo-, Ti-, V- and W-compounds in Balb/3T3 cells (72-hour exposure)

Hard metal	**CFE (% of control ± SEM)**
Control	100
Co	80,6 ± 4.6
Mo	88,3 ± 3.5
Co + Mo + Ti + W	84,9 ± 4.8
Co + V + Mo + Ti + W	0

14 In Vitro Morphological Neoplastic Transformation Balb/3T3 Assay

This section fulfils the last step of the proposed work strategy by applying the *in vitro* morphological neoplastic transformation Balb/3T3 assay.

On the basis of the reported cytotoxicity data, optimal dose-levels are established for evaluating the transformation frequency of metal compounds selected on the basis of their toxicological impact on human health (Section 14.1).

Important findings are derived from the assessment of the carcinogenic potential of Pt- and As-compounds (Sections 14.2 and 14.3). Different responses are obtained depending on the chemical form of the metal, its oxidation state as well as ion complexation.

In this section the results of the cloning of type III foci are also reported (Section 14.4).

14.1 Carcinogenic Potential of Selected Metal Compounds

Table 14.I shows the results of the concurrent cytotoxicity and morphological neoplastic transformation Balb/3T3 assay by exposing the cells for 72 hours to concentrations from 0.1 μM to 700 μM of six metal compounds of group III ($NaAsO_2$, $Na_2HAsO_4 \cdot 7H_2O$, $CdCl_2 \cdot 2H_2O$, $Na_2CrO_4 \cdot 4H_2O$, $(NH_4)_2PtCl_6$, $NaVO_3 \cdot H_2O$); one of group II ($(NH_4)_3RhCl_6$); and three of group I ($CrCl_3 \cdot 6H_2O$, $(NH_4)_2PdCl_6$, $(CH_3)_3AsCH_2COO^-$) (Table 13.I).

The determined transformation frequency (Tf) allowed the ranking of carcinogenic potential to be established:

$CdCl_2 \cdot 2H_2O > NaAsO_2 > NaVO_3 \cdot H_2O > (NH_4)_2PtCl_6 > Na_2HAsO_4 \cdot 7H_2O > Na_2CrO_4 \cdot 4H_2O$.

$(NH_4)_3RhCl_6$, $(NH_4)_2PdCl_6$, $CrCl_3 \cdot 6H_2O$ and $(CH_3)_3AsCH_2COO^-$ were not found transforming. Photos 14.1 and 14.2 illustrate examples of type III foci induced by $(NH_4)_2PtCl_6$ and $NaAsO_2$.

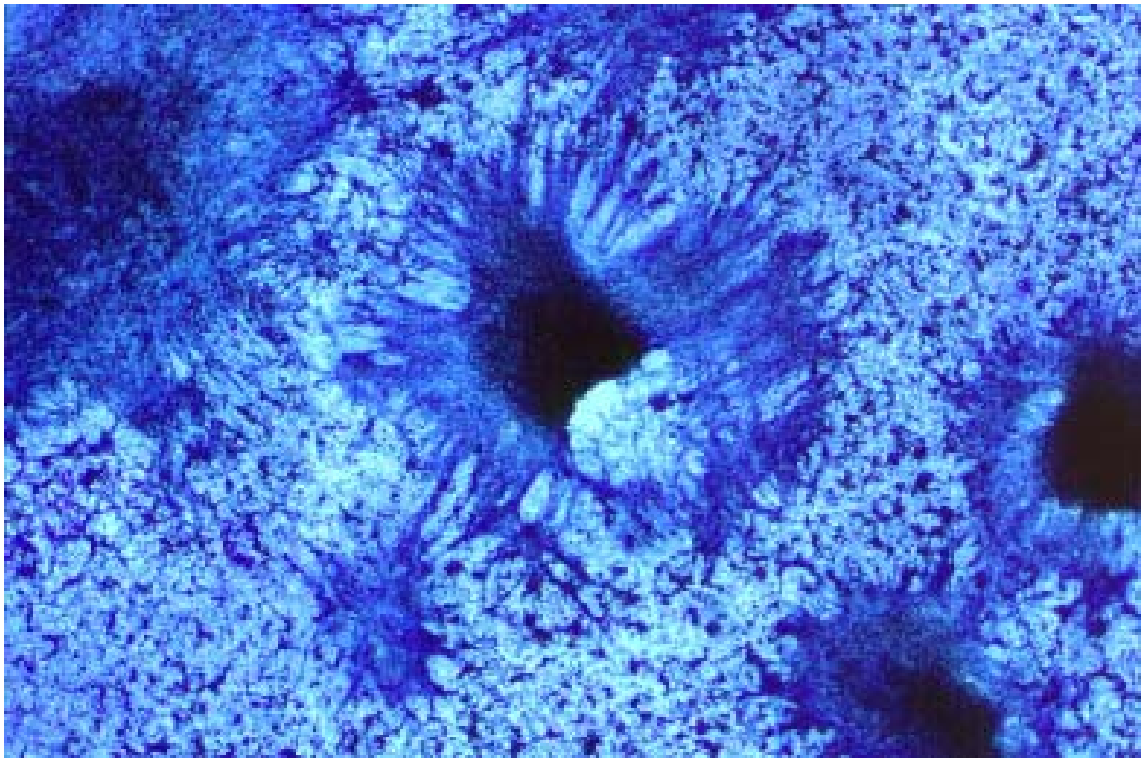
Table 14.I: Concurrent cytotoxicity and morphological neoplastic transformation induced in Balb/3T3 cells by 10 metal compounds

Exposure		CFE (%) ± SEM	N° type III foci / N° dishes	N° type III foci positive dishes / N° dishes	Transforming frequency (T_f) x 10^{-4}
Chemical compound	**Concentration (µM)**				
H_2O biol.	0.1% v/v	100	1 / 20	1 / 20	0.03
B*(a)*P [a]	0.1	10.5 ± 1.5	13 / 20	9 / 20	13.1
$NaAsO_2$	1	78 ± 3	0 / 18	0 / 18	0.0
$NaAsO_2$	3	28 ± 2	2 / 18	2 / 18	0.6
$NaAsO_2$	5	12 ± 1	5 / 18	4 / 18	2.3
$NaAsO_2$	6	5 ± 1	9 / 18	6 / 18	5.7
$Na_2HAsO_4 \cdot 7H_2O$	10	53 ± 1	0 / 18	0 / 18	0.0
$Na_2HAsO_4 \cdot 7H_2O$	20	28 ± 1.5	9 / 18	7 / 18	2.6
$Na_2HAsO_4 \cdot 7H_2O$	30	11 ± 1.5	9 / 18	8 / 18	7.8
$(CH_3)_3AsCH_2COO^-$	50	94 ± 2.5	0 / 18	0 / 18	0.0
$(CH_3)_3AsCH_2COO^-$	100	90 ± 3	0 / 18	0 / 18	0.0
$(CH_3)_3AsCH_2COO^-$	500	94 ± 1.5	0 / 18	0 / 18	0.0
$(CH_3)_3AsCH_2COO^-$	700	91 ± 1.5	0 / 18	0 / 18	0.0
$CdCl_2 \cdot 2H_2O$	1	86 ± 2.5	6 / 20	5 / 20	0.4
$CdCl_2 \cdot 2H_2O$	3	42 ± 1.5	12 / 20	8 / 20	1.6
$CdCl_2 \cdot 2H_2O$	5	27 ± 1	12 / 18	11 / 18	3.6
$CdCl_2 \cdot 2H_2O$	6	16 ± 1.5	17 / 18	15 / 18	9.7

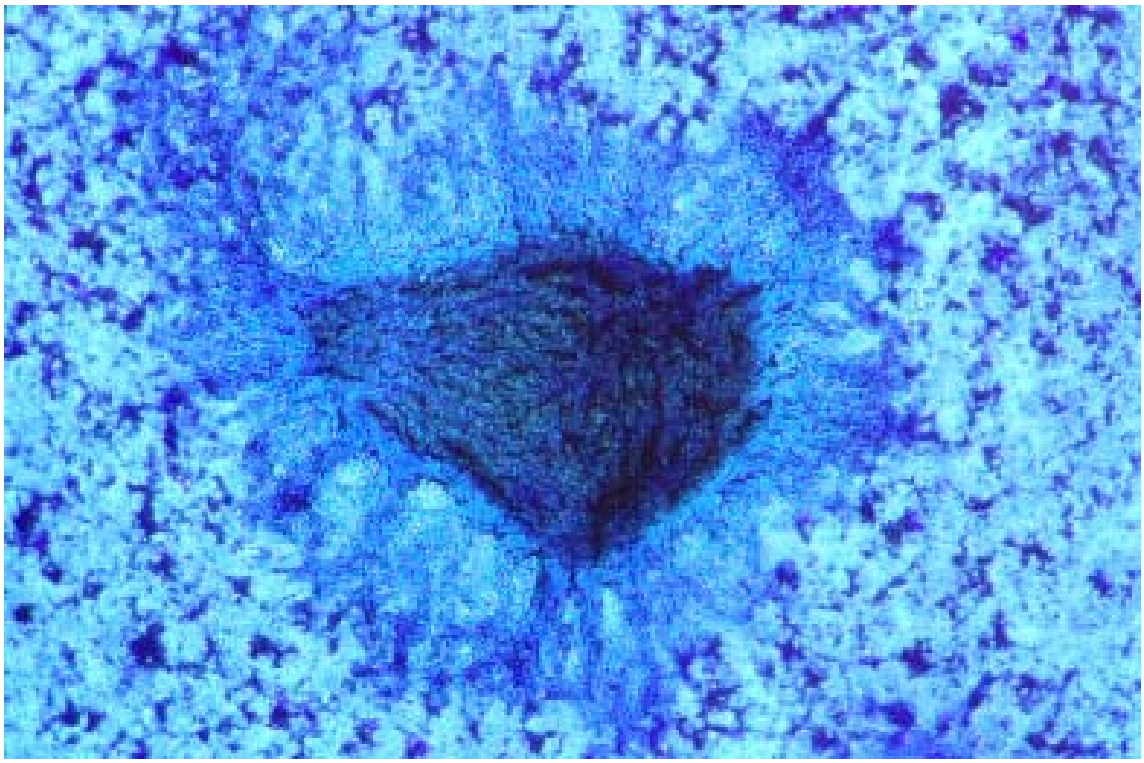
Table 14.I: Continued

Exposure		CFE (%) ± SEM	N° type III foci / N° dishes	N° type III foci positive dishes / N° dishes	Transforming frequency (T_f) x 10^{-4}
Chemical compound	**Concentration (µM)**				
H_2O biol.	0.1% v/v	100	1 / 20	1 / 20	0.03
B*(a)*P [a]	0.1	10.5 ± 1.5	13 / 20	9 / 20	13.1
$CrCl_3 \cdot 6H_2O$	50	88 ± 3	0 / 18	0 / 18	0.0
$CrCl_3 \cdot 6H_2O$	100	92 ± 2	0 / 18	0 / 20	0.0
$CrCl_3 \cdot 6H_2O$	500	89 ± 2.5	1 / 18	1 / 18	0.04
$Na_2CrO_4 \cdot 4H_2O$	10	81 ± 3	2 / 20	2 / 20	0.1
$Na_2CrO_4 \cdot 4H_2O$	30	67 ± 3	19 / 20	12 / 20	2.7
$Na_2CrO_4 \cdot 4H_2O$	50	38 ± 2.5	21 / 20	11 / 20	5.3
$Na_2CrO_4 \cdot 4H_2O$	60	16 ± 1.5	31 / 20	15 / 20	14
$(NH_4)_2PdCl_6$	10	76.3 ± 2.5	0 / 20	0 / 20	0.0
$(NH_4)_2PdCl_6$	100	62.5 ± 2.5	1 / 20	1 / 20	0.15
$(NH_4)_2PtCl_6$	0.1	91.3 ± 3.5	0 / 18	0 / 18	0.0
$(NH_4)_2PtCl_6$	1	74.6 ± 3	2 / 18	2 / 18	0.3
$(NH_4)_2PtCl_6$	5	34.5 ± 2.5	5 / 18	5 / 18	1.75
$(NH_4)_2PtCl_6$	7	19.6 ± 1.5	8 / 18	7 / 18	4.1
$(NH_4)_3RhCl_6$	10	80.6 ± 3	1 / 20	1 / 20	0.15
$(NH_4)_3RhCl_6$	100	57.3 ± 3.5	1 / 20	1 / 20	0.18
$NaVO_3 \cdot H_2O$	1	84 ± 3	2 / 18	2 / 18	1.0
$NaVO_3 \cdot H_2O$	3	64 ± 2.5	9 / 18	6 / 18	2.7
$NaVO_3 \cdot H_2O$	6	27 ± 1.5	11 / 18	8 / 18	5.0

a: positive control.

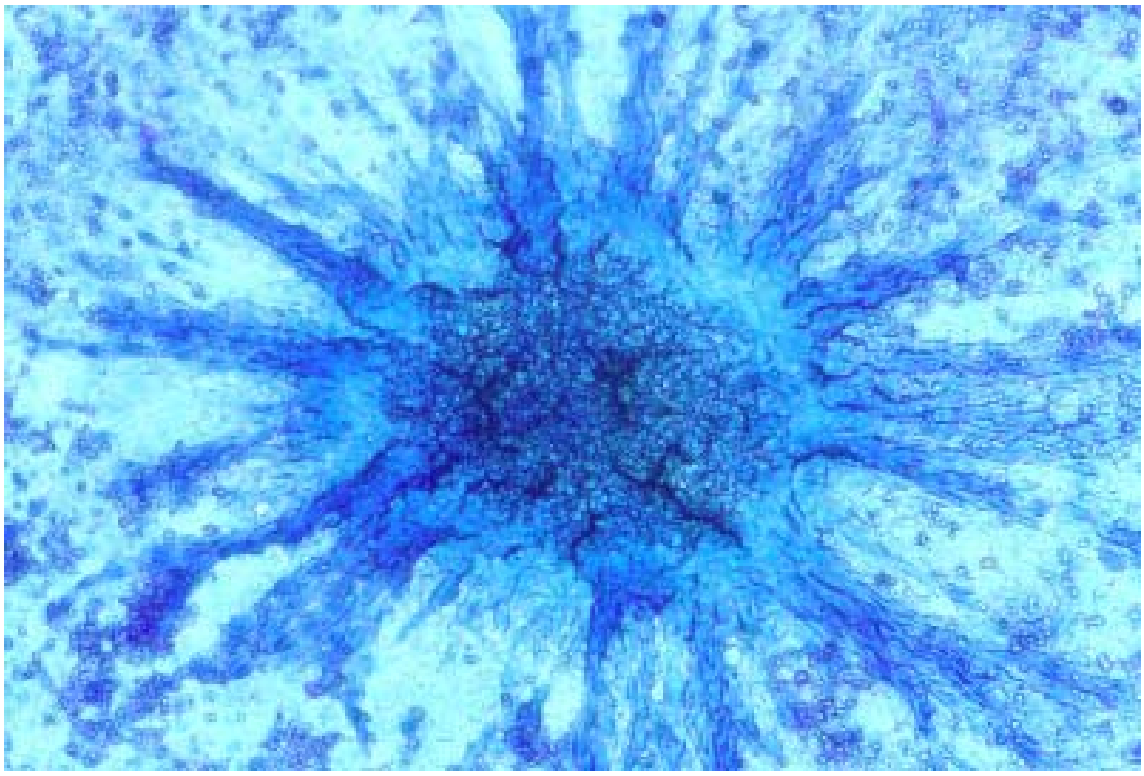


a

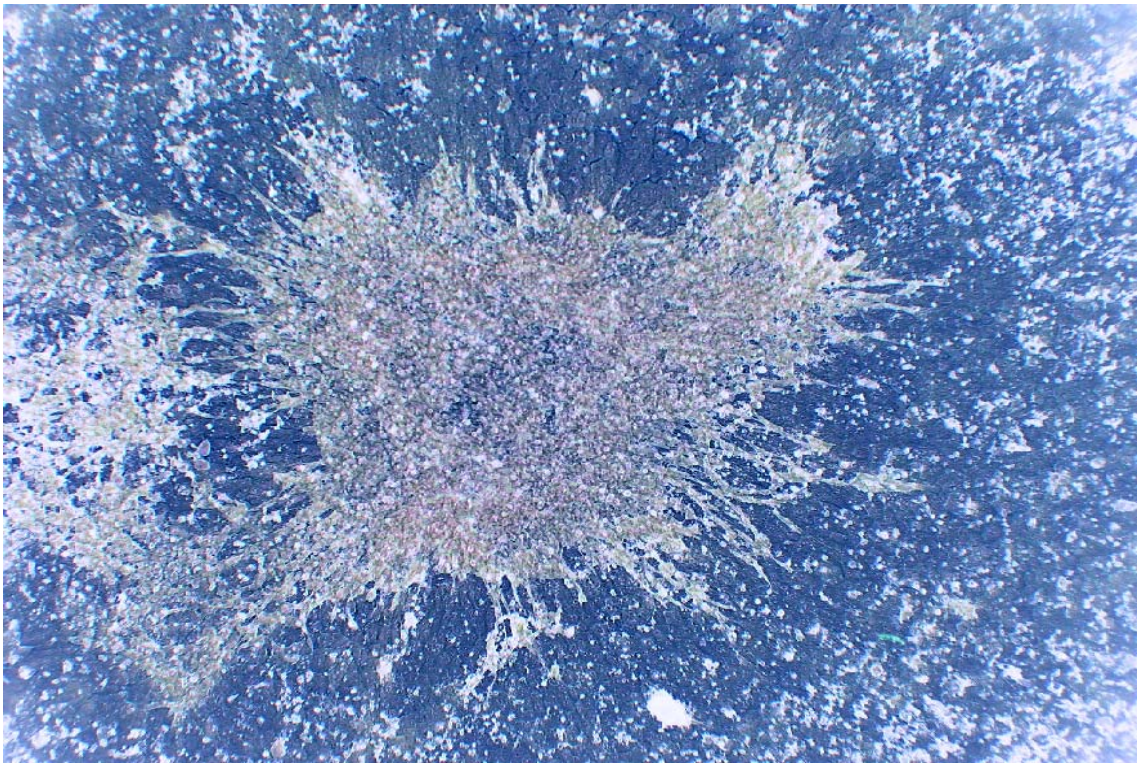


b

Photo 14.1: Two examples of type III transformed foci induced in Balb/3T3 cells exposed to 7 µM $(NH_4)_2PtCl_6$ (a: 100X; b: 200X)



a



b

Photo 14.2: a) type III transformed focus induced in Balb/3T3 cells exposed to 6 µM $NaAsO_2$ (200X)

b) type III transformed focus induced in Balb/3T3 cells exposed to 5 µM $NaAsO_2$ (100X)

14.2 Morphological Transformation of Pt-compounds

Table 14.II reports the results of the morphological transformation of Balb/3T3 cells exposed to $(NH_4)_2PtCl_6$, $(NH_4)_2PtCl_4$, $PtCl_2$, $PtCl_4$, *cis*-Pt and carbo-Pt (from 0.1 μM to 7 μM).

The ranking of transformation response follows the corresponding ranking of the cytotoxicity observed for the same Pt-species (Section 13.3):

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

Table 14.II: Concurrent cytotoxicity and morphological neoplastic transformation induced in Balb/3T3 cells by 5 Pt-compounds (72-hour exposure)

Exposure		CFE (%) ± SEM	N° type III foci / N° dishes	N° type III foci positive dishes / N° dishes	Transforming frequency (T_f) x 10^{-4}
Chemical compound	**Concentration (µM)**				
H_2O biol.	0.1% v/v	100	1 / 20	1 / 20	0.03
B*(a)*P [a]	0.1	10.5 ± 1.5	13 / 20	9 / 20	13.1
$(NH_4)_2PtCl_6$	0.1	91.3 ± 3.5	0 / 18	0 / 18	0.0
$(NH_4)_2PtCl_6$	1	74.6 ± 3	2 / 18	2 / 18	0.3
$(NH_4)_2PtCl_6$	5	34.5 ± 2.5	5 / 18	5 / 18	1.75
$(NH_4)_2PtCl_6$	7	19.6 ± 1.5	8 / 18	7 / 18	4.1
$(NH_4)_2PtCl_4$	1	96.4 ± 2.3	0 / 20	0 / 20	0.0
$(NH_4)_2PtCl_4$	7	89.2 ± 3.3	0 / 20	0 / 20	0.0
$PtCl_2$	0.1	97.7 ± 3.1	3 / 20	3 / 20	0.3
$PtCl_2$	0.3	85.7 ± 2.9	8 / 20	7 / 20	0.8
$PtCl_2$	0.5	65.8 ± 2.1	14 / 20	8 / 20	1.6
$PtCl_2$	0.7	50.9 ± 2.4	38 / 20	14 / 20	6.8
$PtCl_4$	7	14.8 ± 2.0	24 / 20	8 / 20	7.5
cis-Pt	0.5	64.4 ± 1.9	43 / 20	10 / 20	5.8
cis-Pt	0.7	69.7 ± 3.4	64 / 18	15 / 20	29.1
cis-Pt	1	1.5 ± 1.6	82 / 20	15 / 20	174.5
carbo-Pt	0.7	85.1 ± 3.8	13 / 20	6 / 20	0.9
carbo-Pt	1	75.1 ± 4	13 / 20	7 / 20	1.0
carbo-Pt	3	24.8 ± 3	45 / 20	15 / 20	19

a: positive control.

14.3 Morphological Transformation of Organoarsenic Compounds

Table 14.III presents the results of morphological transformation of Balb/3T3 cells exposed to three organoarsenic compounds: $(CH_3)_3AsCH_2COO^-$, $((CH_3)_3AsCH_2CH_2OH)Br$ and $(C_5H_6)_4AsCl{\cdot}H_2O$. In the range of concentrations tested (from 3 µM to 700 µM), only $(C_5H_6)_4AsCl{\cdot}H_2O$ was transforming in a dose-response fashion.

Table 14.III: Concurrent cytotoxicity and morphological neoplastic transformation induced in Balb/3T3 cells by 3 selected organoarsenic compounds

Exposure		CFE (%) ± SEM	N° type III foci / N° dishes	N° type III foci positive dishes / N° dishes	Transforming frequency (T_f) x 10^{-4}
Chemical compound	**Concentration (µM)**				
H_2O biol.	0.1% v/v	100	1 / 20	1 / 20	0.03
B*(a)*P [a]	0.1	10.5 ± 1.5	13 / 20	9 / 20	13.1
$(CH_3)_3AsCH_2COO^-$	50	94 ± 2.5	0 / 18	0 / 18	0.0
$(CH_3)_3AsCH_2COO^-$	100	90 ± 3	0 / 18	0 / 18	0.0
$(CH_3)_3AsCH_2COO^-$	500	94 ± 1.5	0 / 18	0 / 18	0.0
$(CH_3)_3AsCH_2COO^-$	700	91 ± 1.5	0 / 18	0 / 18	0.0
$((CH_3)_3AsCH_2CH_2OH)Br$	10	91.5 ± 1.5	0 / 20	0 / 20	0.0
$((CH_3)_3AsCH_2CH_2OH)Br$	100	90.6 ± 2.0	0 / 20	0 / 20	0.0
$(C_6H_5)_4AsCl{\cdot}H_2O$	3	74.6 ± 3	1 / 20	1 / 20	0.2
$(C_6H_5)_4AsCl{\cdot}H_2O$	5	61.7 ± 2	2 / 20	1 / 20	0.5
$(C_6H_5)_4AsCl{\cdot}H_2O$	7	12.8 ± 2.5	11/ 20	6 / 20	5.9

a: positive control.

14.4 Cloning of Type III Foci and Soft Agar

At the end of morphological transformation assay carried out on $NaAsO_2$, $CdCl_2 \cdot 2H_2O$, *cis*-Pt, carbo-Pt, $(NH_4)_2PtCl_6$, $PtCl_4$ and $PtCl_2$, some culture dishes were not fixed and stained but selected in order to clone type III foci (Section 4.6).

Interestingly, during the amplification of these clones, some of them lost the typical fibroblast-like shape and appeared more spindle-shaped and even epithelial-like (Photo 14.3).

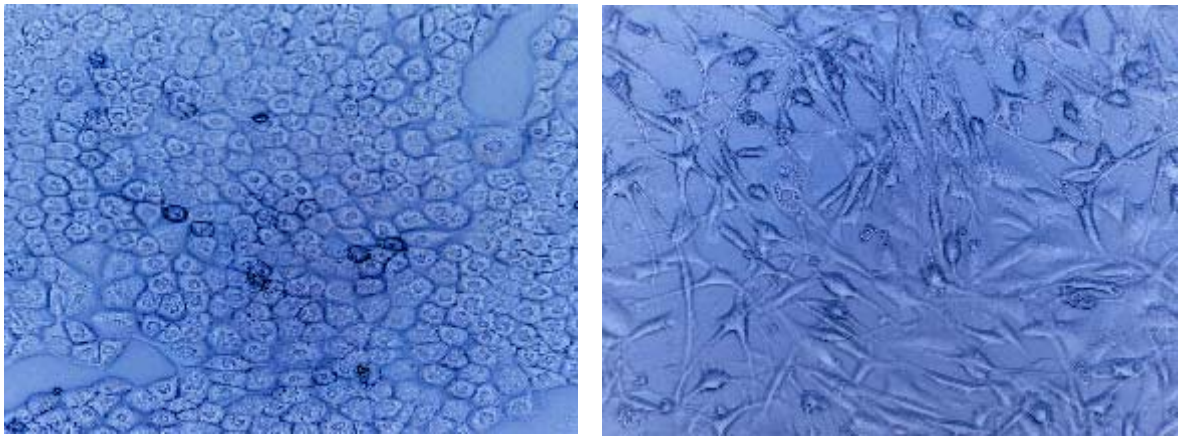
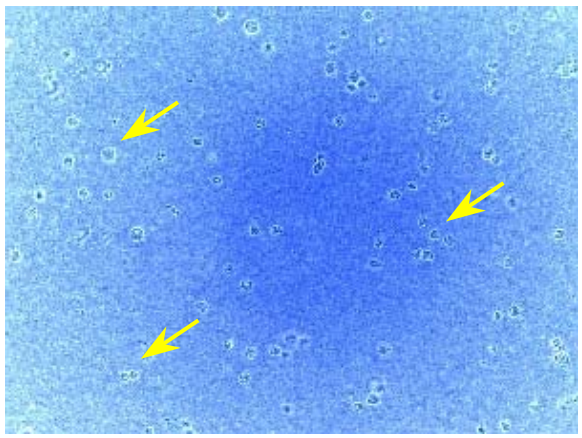


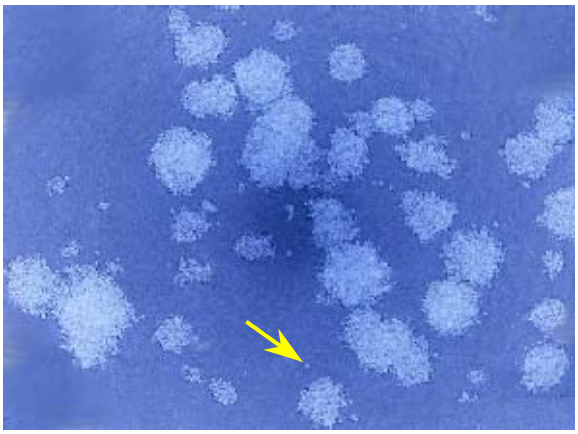
Photo 14.3: Two atypical forms of Balb/3T3 cells after cloning (400X)

In collaboration with the "Istituto Zooprofilattico Sperimentale dell'Emilia e della Lombardia", Brescia (Italy) in order to assess the tumorigenicity of these clones, the assay for anchorage-independent growth using the standard soft agar plating method was applied (Section 4.7). All clones inducing colonies were examined twice to confirm the results. For the Balb/3T3 cells exposed to 6 µM $NaAsO_2$, 5 µM $CdCl_2 \cdot 2H_2O$, 1 µM *cis*-Pt, 1 µM and 3 µM carbo-Pt, 5 µM and 7 µM $(NH_4)_2PtCl_6$, 7 µM $PtCl_4$, 0.5 µm $PtCl_2$, clones showed colony formation in agar comparable to the positive control, a spontaneously transformed new-born swine kidney (NSK cells) isolated cell line

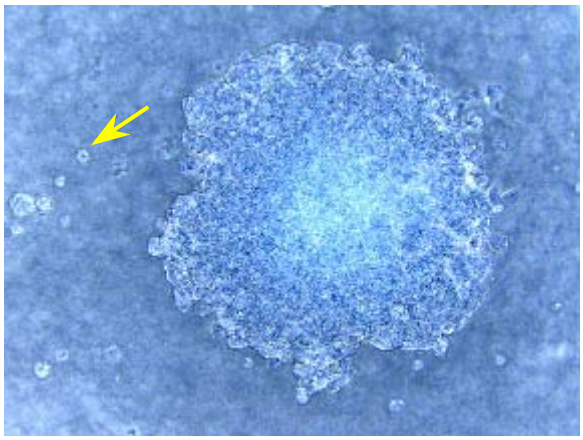
Photo 14.4 shows examples of colony formation induced by $NaAsO_2$ and $(NH_4)_2PtCl_6$ in comparison to negative and positive controls.



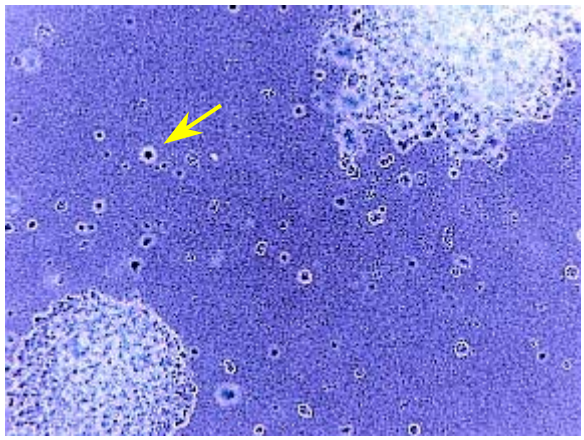
a



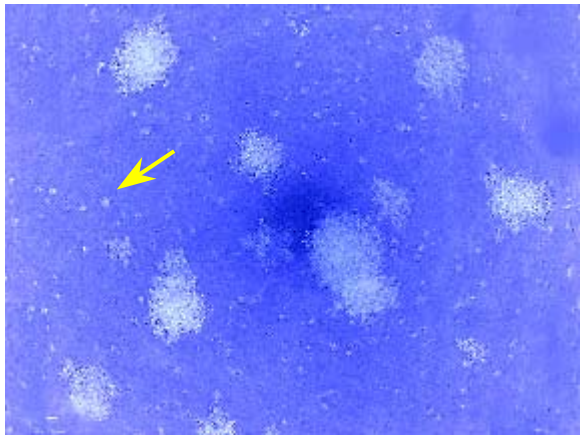
b



c



d



e

Photo 14.4 related to soft agar cultures. ***a:*** *Control negative of Balb/3T3 cells (200X);* ***b:*** *Control positive of NSK cells (200X);* ***c:*** *Particular of colony formation induced by 7µM of* $(NH_4)_2PtCl_6$ *(400X);* ***d:*** *Colony formation induced by 6µM of* $NaAsO_2$ *(400X);* ***e:*** *Colony formation induced by 5µM of* $(NH_4)_2PtCl_6$ *(200X). Single non-dividing cells are arrowed.*