15 Metabolic Studies

This section reports the results of metabolic studies carried out on the Balb/3T3 cell line in order to establish correlation between cytotoxic effects and metabolism of the metal incorporated into cells that are of fundamental importance for mechanistic understanding. An advanced spectrochemical analytical technique, the ICP-MS, was applied in this section to assess Pt-content in cells (Section 15.1).

Furthermore, radiotracers were successfully used in order to follow intracellular repartition and cellular uptake of As-compounds as investigated by ^{73}As radiotracer (Section 15.2) as well as biotransformation of Cr(VI) as determined by ^{51}Cr radioisotope (Section 15.3).

15.1 Uptake of Pt-compounds

Table 15.I shows the results of the cellular uptake of Pt (expressed as fg Pt/cell) as determined by ICP-MS (Section 7.1) after 72-hour exposure of the Balb/3T3 cell line to the following Pt-compounds: inorganic/anionic ($(NH_4)_2PtCl_6$) and inorganic/cationic ($PtCl_2$, $PtCl_4$) species; inorganically complexed ions (*cis*-Pt); and organometallic forms (carbo-Pt). The concentrations tested for each species correspond to those inducing about 80%, 50% and 20% of CFE as determined in previous dose-effect studies (Section 13.3).

The results showed an increase of cellular incorporation of Pt for the assayed Pt-compounds, which was dependent on the exposure concentration and the oxidation state of the Pt-compound tested.

Table 15.I: Uptake of Pt by Balb/3T3 cells

Concentration (µM)	Pt-content (fg/cell ± SEM) [a]
$(NH_4)_2PtCl_6$	
Control	<0.01 ± 0.2
1	2.1 ± 0.5
5	8.2 ± 1.1
10	26.9 ± 1.3
***cis*-Pt**	
Control	<0.01 ± 0.2
0.1	0.08 ± 0.2
0.5	0.86 ± 0.7
0.7	1.01 ± 0.5
carbo-Pt	
Control	<0.01 ± 0.2
0.5	0.35 ± 0.6
1	0.71 ± 1.3
3	2.82 ± 1.4
$PtCl_4$	
Control	<0.01 ± 0.2
0.1	0.07 ± 1.0
3	0.53 ± 0.8
7	1.05 ± 2.1
$PtCl_2$	
Control	<0.01 ± 0.2
0.1	0.22 ± 1.2
0.5	1.6 ± 0.6
0.7	16.83 ± 1.6

a: average of 3 experiments.

15.2 Uptake and Intracellular Repartition of Arsenic in Balb/3T3 Cells

Table 15.II shows the results of the uptake of As in the Balb/3T3 cells after 1- and 24-hour exposure to different concentrations of $Na[^{73}As]AsO_2$ or $Na_2H[^{73}As]AsO_4$. At equimolar concentration (3 μM) cellular uptake of As was about 4-fold higher for As(III) than As(V) at two selected exposure times.

Table 15.II: Uptake of As by Balb/3T3 cells

Concentration (μM)	Uptake (pmoles / 10^6 cells / h ± SEM) [a]			
	As(III)		As(V)	
	1h	24h	1h	24h
0.6	10.6 ± 1.1	0.6 ± 0.1	–	–
1	19.8 ± 2.5	2.3 ± 0.6	–	–
3	42.1 ± 1.6	7.3 ± 1.0	11.2 ± 1.9	1.8 ± 0.4
10	–	–	85.0 ± 3.2	8.8 ± 2.0
30	–	–	127.1 ± 3.0	12.0 ± 1.6

a: average of 3 experiments.

The corresponding intracellular repartition of As (Table 15.III) shows that at non-toxic concentrations (0.1 µM for As(III) and 3 µM for As(V)) more than 95% of As was present in the cytosol fraction for both the As-species tested. At toxic concentrations (3 µM $NaAsO_2$ and 30 µM $Na_2HAsO_4·7H_2O$) about 20% of the intracellular As was found in the cellular organelles as As(III) at 1 h and 24 h, while the corresponding value for the pentavalent form was about 10%.

Table 15.III: Intracellular repartition of As in Balb/3T3 cells

$NaAsO_2$				
Exposure (h)	**As content (% of the total uptake ± SEM) [a]**			
	0.1 µM		**3 µM**	
	Pellet	**Cytosol**	**Pellet**	**Cytosol**
1	5.0 ± 1.5	95.0 ± 1.0	22.0 ± 1.5	78.0 ± 3.5
24	4.0 ± 1.0	96.0 ± 1.0	19.0 ± 2.0	81.0 ± 2.5

$Na_2HAsO_4·7H_2O$				
Exposure (h)	**As content (% of the total uptake ± SEM) [a]**			
	3 µM		**30 µM**	
	Pellet	**Cytosol**	**Pellet**	**Cytosol**
1	3.0 ± 0.5	97.0 ± 2.0	6.0 ± 1.5	94.0 ± 2.5
24	3.0 ± 0.5	97.0 ± 1.0	10.0 ± 1.5	90.0 ± 2.0

a: average of 3 experiments.

15.3 Chromium Speciation in Culture Medium

Table 15.IV shows the results concerning the change of the oxidation state of Cr in DMEM culture medium after exposure from 1 h to 72 h of the Balb/3T3 cells to 1 μM of $Na_2[^{51}Cr]CrO_4$.

The results showed a biotransformation of the initial Cr(VI) to Cr(III) in culture medium. This process appears to be linear with the exposure time, 89.5% being transformed to Cr(III) after 72 hours. Previous experiments on the incubation of Cr(VI) with a cell-free for 72 h showed that no change of the chemical form occurred during the incubation period, 98.5 % of Cr still being in the hexavalent state at the end of the experiment (data not shown).

Table 15.IV: Biotransformation of Cr(VI) to Cr(III) in DMEM after incubation of Balb/3T3 cells with $Na_2CrO_4{\cdot}4H_2O$ from 1 h to 72 h

Incubation time (h)	**^{51}Cr/dish (% of dose ± SEM) [a]**
	Cr(III)
1	31.1 ± 1.7
24	45.1 ± 1.4
48	75.6 ± 1.9
72	89.5 ± 2.8

a: average of 3 experiments.

16 Studies on Apoptosis

In the present section seven metal compounds ($NaAsO_2$, $Na_2CrO_4 \cdot 4H_2O$, *cis*-Pt, carbo-Pt, $(NH_4)_2PtCl_6$, $PtCl_4$, and $PtCl_2$) were investigated for their ability to induce apoptosis in the Balb/3T3 cells exposed to different concentrations for 2, 6, 12, and 24 hours.

Since under these experimental conditions no cytotoxicity data of these metal compounds were available, the MTT test (Section 4.4) was performed in order to correlate the results obtained from the apoptotic study with those concerning the cytotoxic effect.

16.1 Application of Annexin V/PI Assay

Figures 16.1-16.14 show the results obtained at different concentrations and exposure times of $NaAsO_2$, $Na_2CrO_4 \cdot 4H_2O$, *cis*-Pt, carbo-Pt, $(NH_4)_2PtCl_6$, $PtCl_4$ and $PtCl_2$, by exploiting the ability of the annexin V/PI assay to detect early stages of apoptosis in a sample of suspended cells (Section 9.1).

For each metal compound the elaboration of the cytograms (Figure 9.1*b*) is presented as curves (results reached for each concentration tested over all exposure times considered) and histograms (living, apoptotic or necrotic cell populations at different exposure times).

Arsenic. The apoptotic response induced by $NaAsO_2$ ranged from 8.9% at 50 µM to 32.6% at 175 µM after 6-hour exposure. At 50 µM the response was still positive after 12 hours, while already after 6 hours at 200 µM the necrosis value was higher than apoptosis (Figures 16.1-16.2).

Table 16.I shows the cytotoxic effect induced in the Balb/3T3 cells by $NaAsO_2$ over the range of concentrations and exposure times considered.

Table 16.I: Cytotoxicity induced by $NaAsO_2$ in Balb/3T3 cells

Concentration (µM)	**MTT (% of control ± SEM)**			
	Exposure (h)			
	2	**6**	**12**	**24**
50	100.0 ± 1.0	81.7 ± 2.0	75.1 ± 4.1	32.0 ± 0.3
100	96.9 ± 2.0	82.8 ± 2.2	21.9 ± 0.5	2.2 ± 0.4
150	80.4 ± 9.5	74.2 ± 0.8	20.4 ± 0.6	2.5 ± 0.5
175	88.4 ± 3.4	70.2 ± 1.5	19.0 ± 0.5	3.2 ± 0.2
200	85.9 ± 4.2	66.4 ± 1.3	15.5 ± 0.4	3.1 ± 0.4

Figure 16.1: Curves of living, apoptosis and necrosis in Balb/3T3 cells exposed to $NaAsO_2$

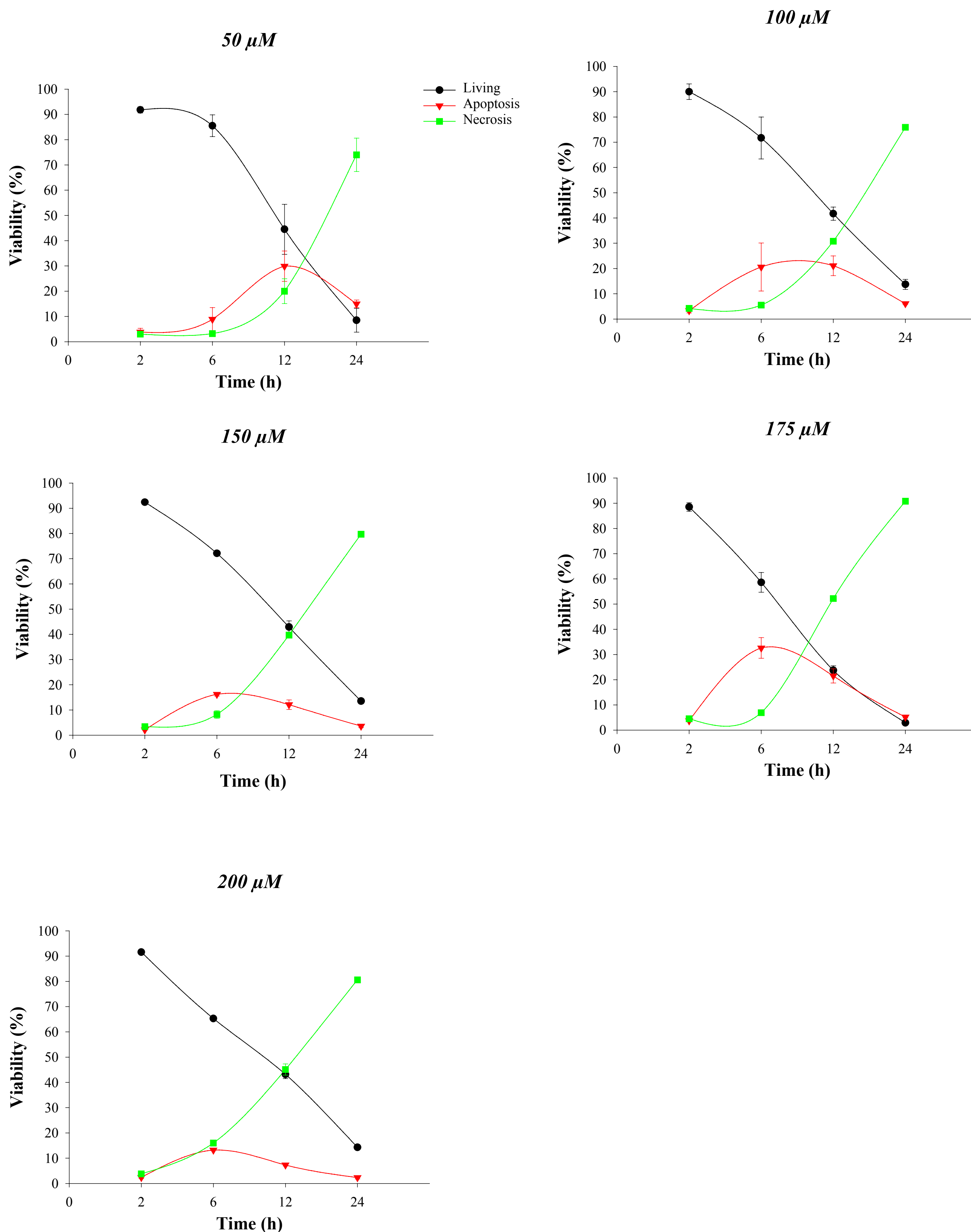
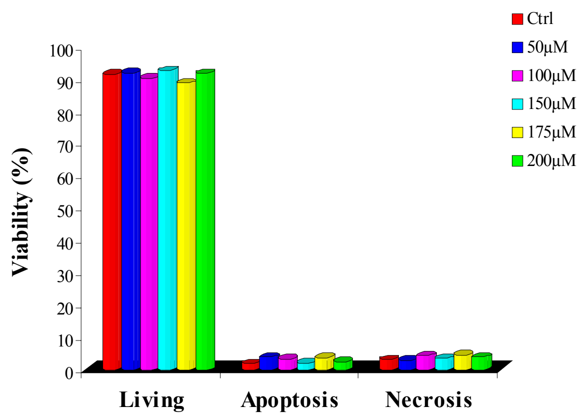
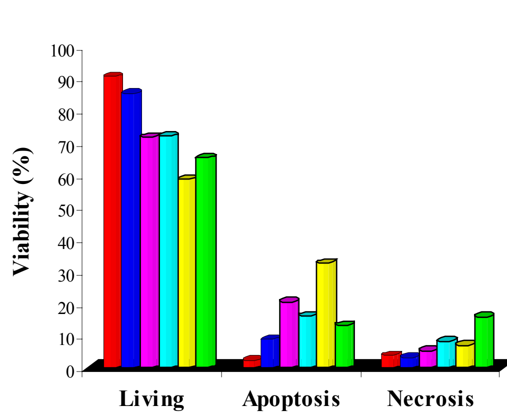


Figure 16.2: Histograms of living, apoptosis and necrosis in Balb/3T3 cells exposed to $NaAsO_2$

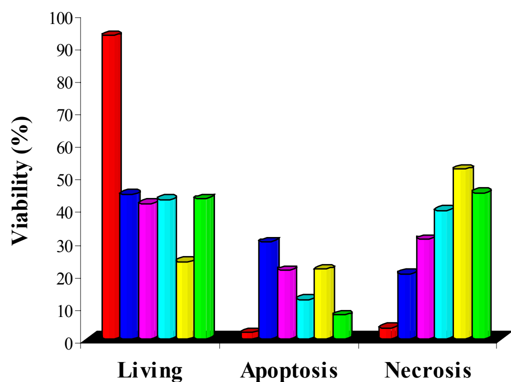
2 hours



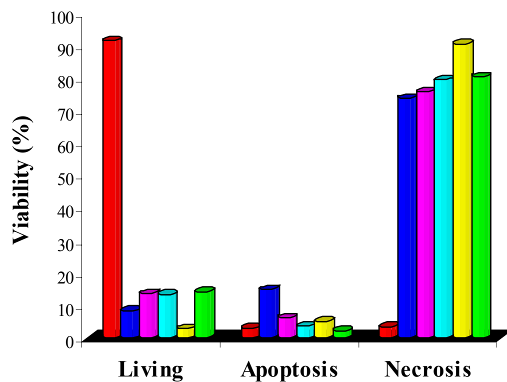
6 hours



12 hours



24 hours



Chromium. $Na_2CrO_4{\cdot}4H_2O$ induced apoptosis after 6-hour exposure at all concentrations tested, ranging from 13.8% at 250 μM to 21.2% at 350 μM (Figures 16.3-16.4).

Table 16.II shows the cytotoxic response to Cr(VI) over the range of concentrations and exposure times considered.

Table 16.II: Cytotoxicity induced by $Na_2CrO_4{\cdot}4H_2O$ in Balb/3T3 cells

Concentration (μM)	**MTT (% of control ± SEM)**			
	Exposure (h)			
	2	**6**	**12**	**24**
250	61.0 ± 1.7	36.7 ± 0.4	8.7 ± 0.5	0.0
300	67.4 ± 2.4	33.5 ± 0.5	7.5 ± 0.4	0.0
350	66.9 ± 1.1	30.0 ± 0.5	6.1 ± 0.2	0.0

Figure 16.3: Curves of living, apoptosis and necrosis in Balb/3T3 cells exposed to $Na_2CrO_4 \cdot 4H_2O$

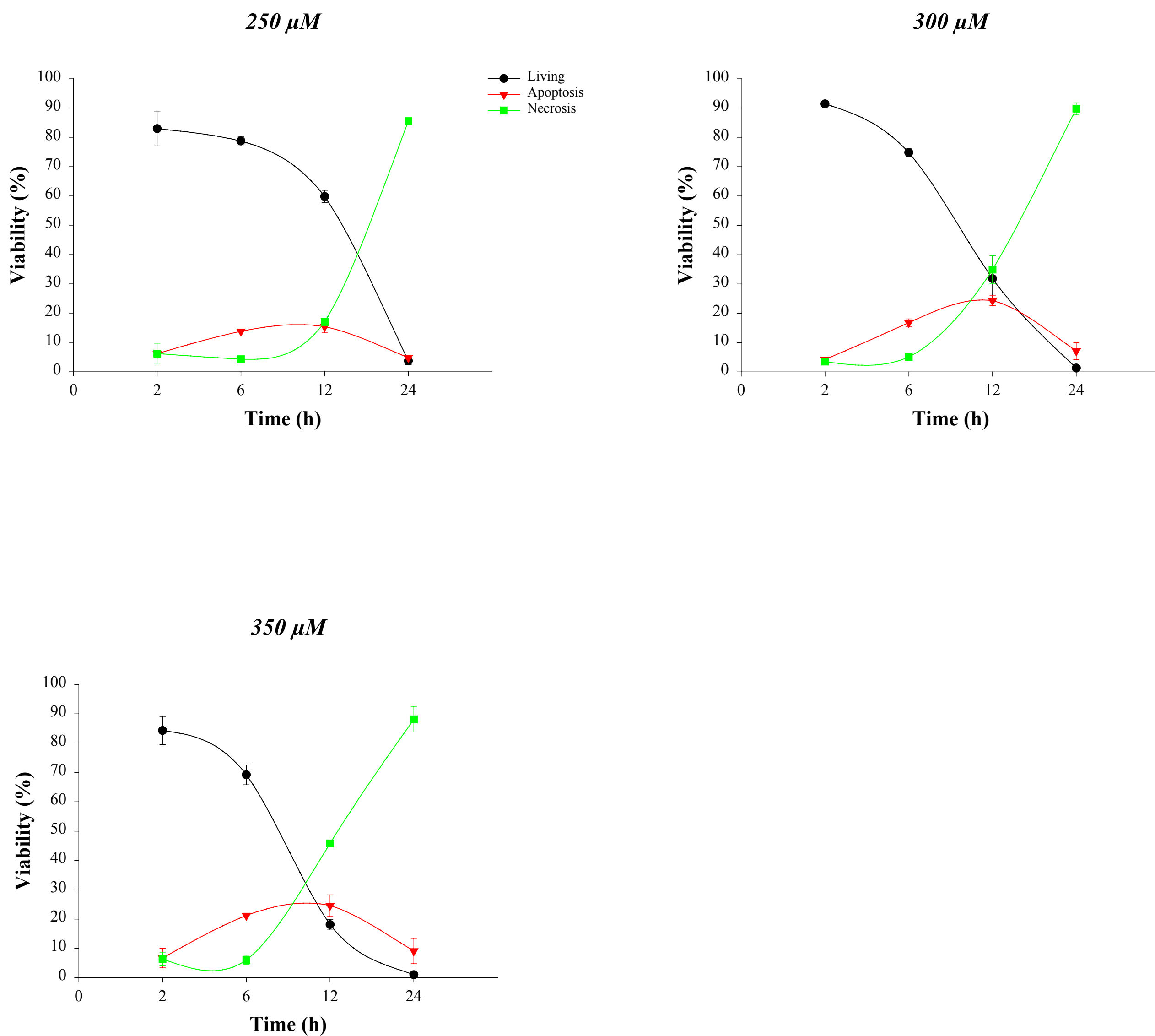
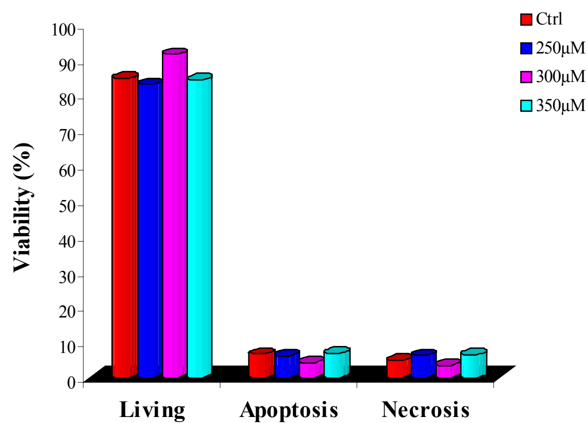
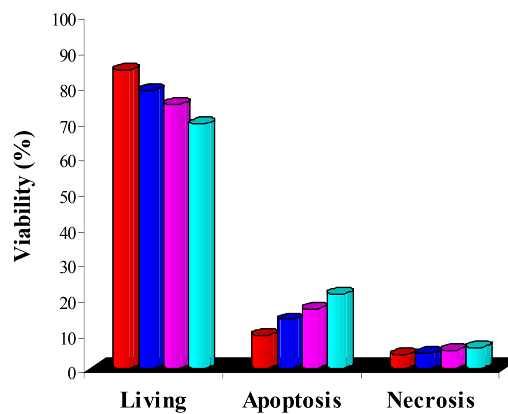


Figure 16.4: Histograms of living, apoptosis and necrosis in Balb/3T3 cells exposed to $Na_2CrO_4 \cdot 4H_2O$

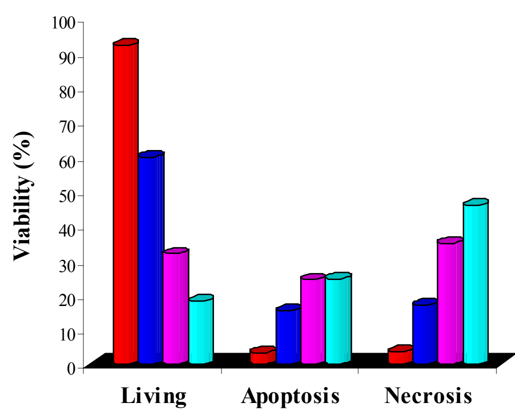
2 hours



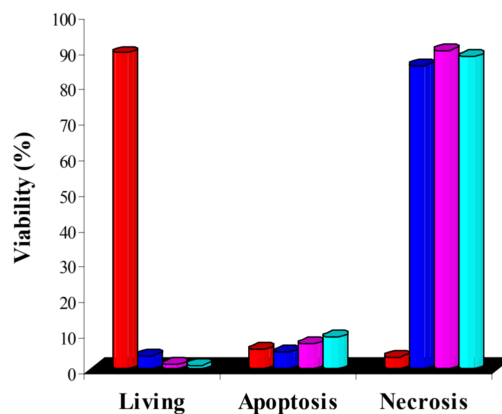
6 hours



12 hours



24 hours



Cis-Pt. After 12-hour exposure *cis*-Pt showed an apoptotic response at 85, 100 and 150 μM ranging from 29.9% to 42.4%. At 85 and 100 μM still 50% of cells were living. At 200 μM after 12-hour exposure necrosis was higher than apoptosis (Figures 16.5-16.6).

Data concerning the related cytotoxic effect of *cis*-Pt are reported in Table 16.III.

Table 16.III: Cytotoxicity induced by cis-Pt in Balb/3T3 cells

Concentration (μM)	MTT (% of control ± SEM)			
	Exposure (h)			
	2	6	12	24
85	101.9 ± 3.4	90.4 ± 2.4	89.1 ± 5.1	15.9 ± 3.8
100	105.8 ± 1.7	87.7 ± 2.8	83.6 ± 0.7	10.8 ± 1.2
150	95.9 ± 3.8	87.9 ± 2.3	27.2 ± 1.0	0.0
200	91.0 ± 5.1	86.7 ± 5.6	10.3 ± 0.6	0.0

Figure 16.5: Curves of living, apoptosis and necrosis in Balb/3T3 cells exposed to cis-Pt

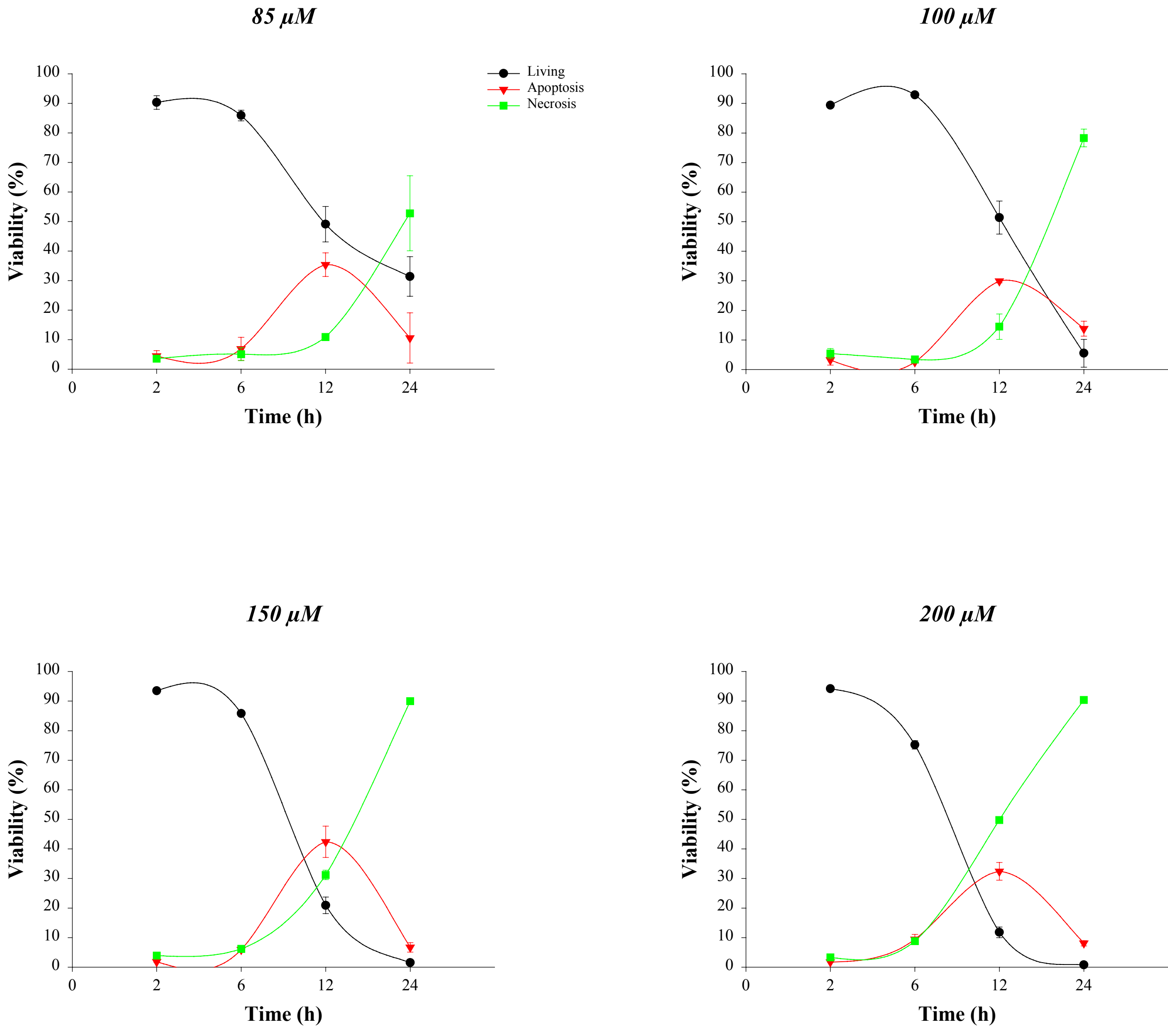
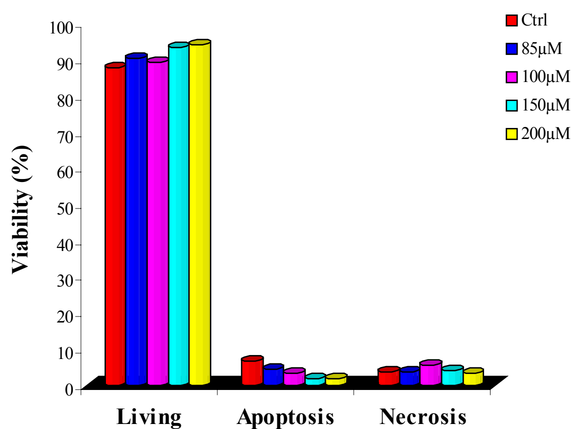
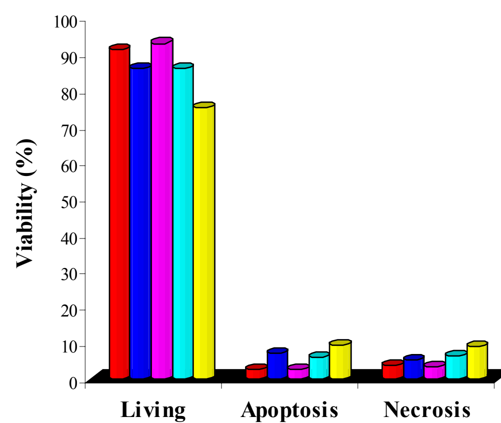


Figure 16.6: Histograms of living, apoptosis and necrosis in Balb/3T3 cells exposed to cis-Pt

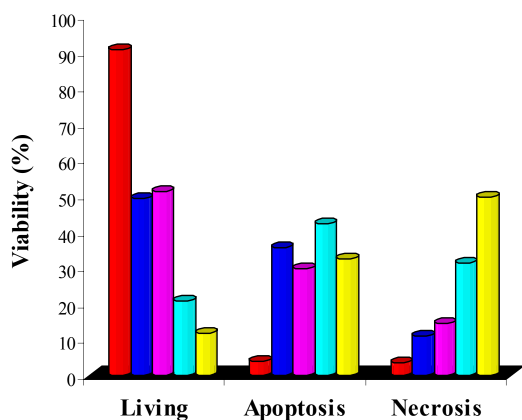
2 hours



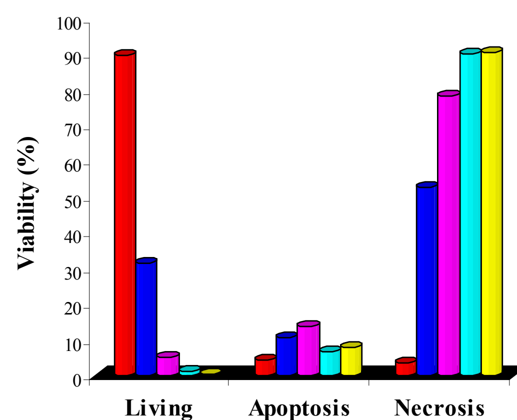
6 hours



12 hours



24 hours



Carbo-Pt. Apoptosis in the Balb/3T3 cells exposed to concentrations of carbo-Pt from 1000 to 2000 μM ranged from 16.8% (1000 μM) to 46.9% (1500 μM) after 12-hour exposure. Only after 24 hours there was a clear increase of the necrotic values (Figures 16.7-16.8).

Table 16.IV shows the results referring to the cytotoxic effect induced by carbo-Pt at the concentrations tested and after the exposure times considered.

Table 16.IV: Cytotoxicity induced by carbo-Pt in Balb/3T3 cells

Concentration (μM)	**MTT (% of control ± SEM)**			
	Exposure (h)			
	2	**6**	**12**	**24**
1000	104.3 ± 4.1	98.6 ± 1.6	101.4 ± 1.6	50.3 ± 2.8
1250	101.3 ± 5.1	96.4 ± 2.0	101.5 ± 2.2	29.1 ± 6.7
1500	100.2 ± 3.9	99.6 ± 2.5	95.2 ± 1.7	1.4 ± 0.5
1750	104.3 ± 5.6	99.9 ± 2.9	86.7 ± 0.7	0.0
2000	100.4 ± 3.5	99.2 ± 1.9	67.4 ± 0.7	0.0

Figure 16.7: Curves of living, apoptosis and necrosis in Balb/3T3 cells exposed to carbo-Pt

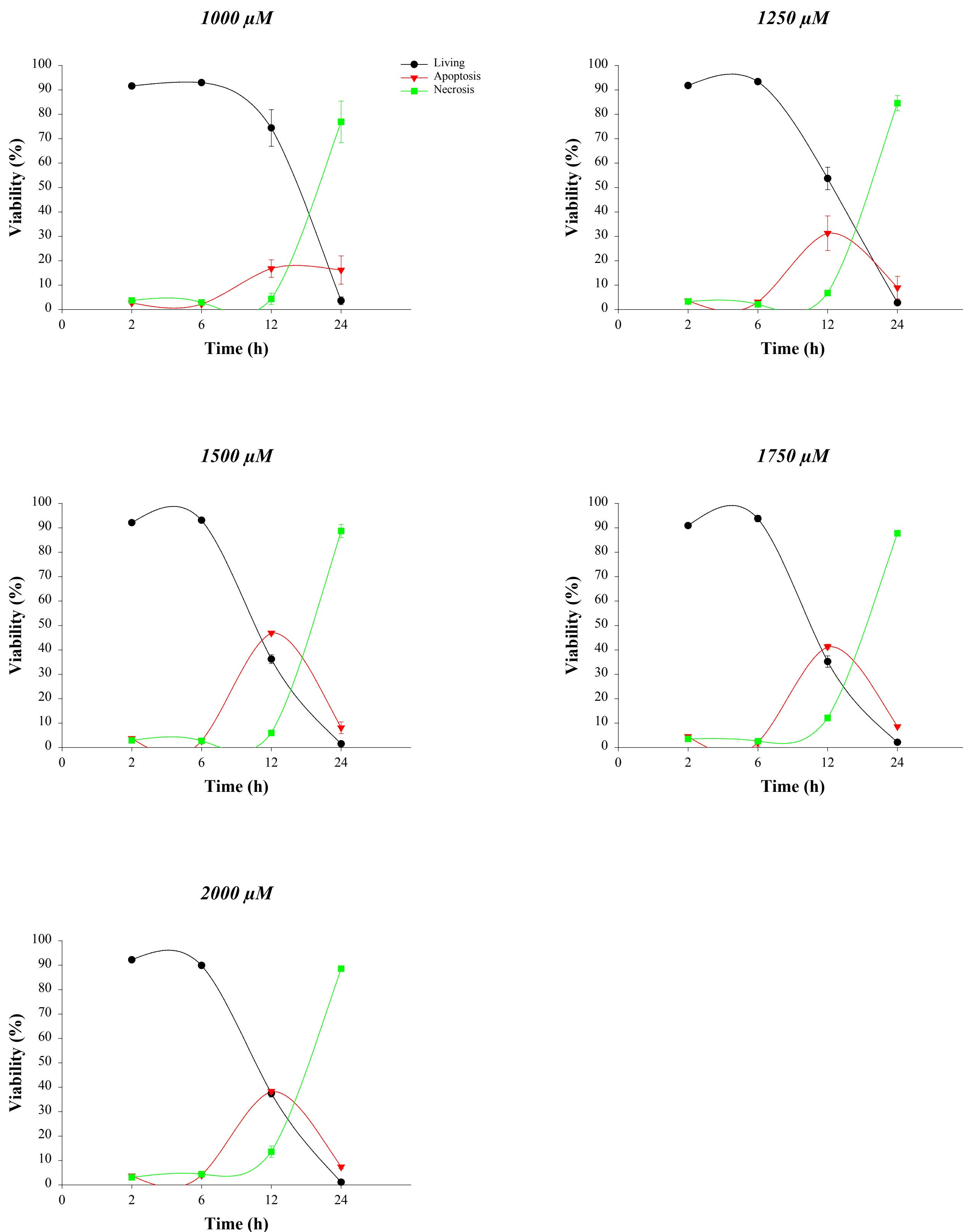
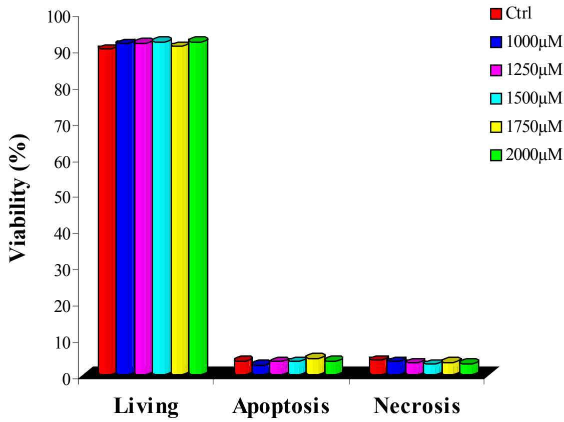
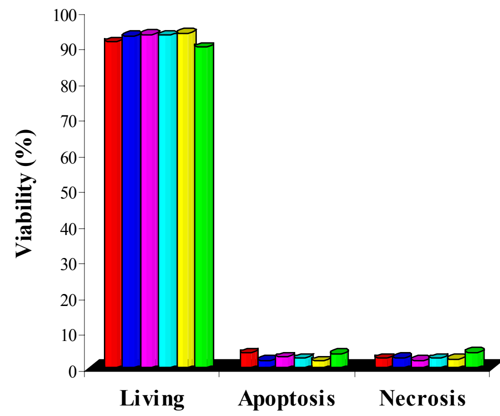


Figure 16.8: Histograms of living, apoptosis and necrosis in Balb/3T3 cells exposed to carbo-Pt

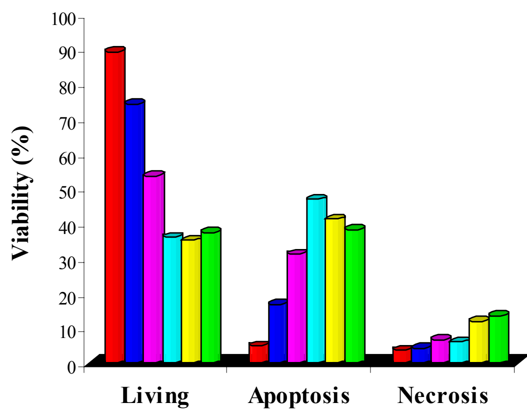
2 hours



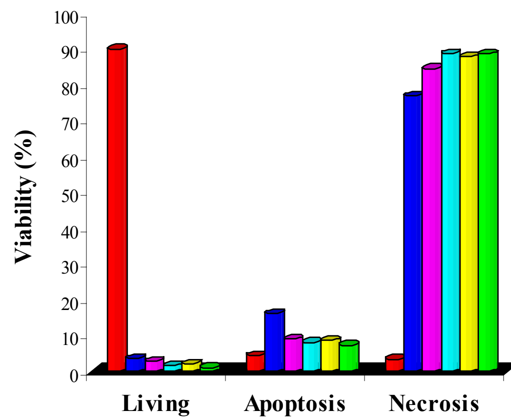
6 hours



12 hours



24 hours



<u>$(NH_4)_2PtCl_6$, $PtCl_4$ and $PtCl_2$.</u> The study carried out on $(NH_4)_2PtCl_6$, $PtCl_4$ and $PtCl_2$ did not indicate a significant induction of apoptosis (Figures 16.9-16.10, 16.11-16.12, 16.13-16.14, respectively). Over the whole range of concentrations and exposure times tested, a very strong predominance of necrosis was detected.

Table 16.V reports the related cytotoxicity data.

Table 16.V: Cytotoxicity induced by $(NH_4)_2PtCl_6$, $PtCl_4$ and $PtCl_2$ in Balb/3T3 cells

Concentration (µM)	**MTT (% of control ± SEM)**			
	Exposure (h)			
	2	**6**	**12**	**24**
	$(NH_4)_2PtCl_6$			
50	95.0 ± 2.3	100.1 ± 2.3	85.3 ± 2.8	95.8 ± 2.8
75	83.3 ± 3.5	95.4 ± 1.9	74.7 ± 3.9	71.8 ± 0.1
100	76.5 ± 3.0	92.8 ± 1.6	74.1 ± 2.9	47.7 ± 4.8
	$PtCl_4$			
100	89.1 ± 5.4	86.1 ± 2.7	88.0 ± 1.6	79.8 ± 1.3
150	85.7 ± 2.9	81.4 ± 1.6	65.6 ± 1.3	44.6 ± 2.4
175	82.8 ± 3.4	76.2 ± 1.1	58.0 ± 1.5	26.0 ± 1.4
200	78.6 ± 3.7	75.1 ± 1.5	47.1 ± 1.1	17.6 ± 1.1
	$PtCl_2$			
50	92.4 ± 2.0	91.6 ± 4.1	55.5 ± 1.2	40.6 ± 4.1
75	90.4 ± 4.4	84.2 ± 3.2	34.2 ± 0.7	20.9 ± 3.0
100	73.8 ± 3.7	63.3 ± 2.8	24.5 ± 0.4	12.3 ± 1.7

Figure 16.9: Curves of living, apoptosis and necrosis in Balb/3T3 cells exposed to $(NH_4)_2PtCl_6$

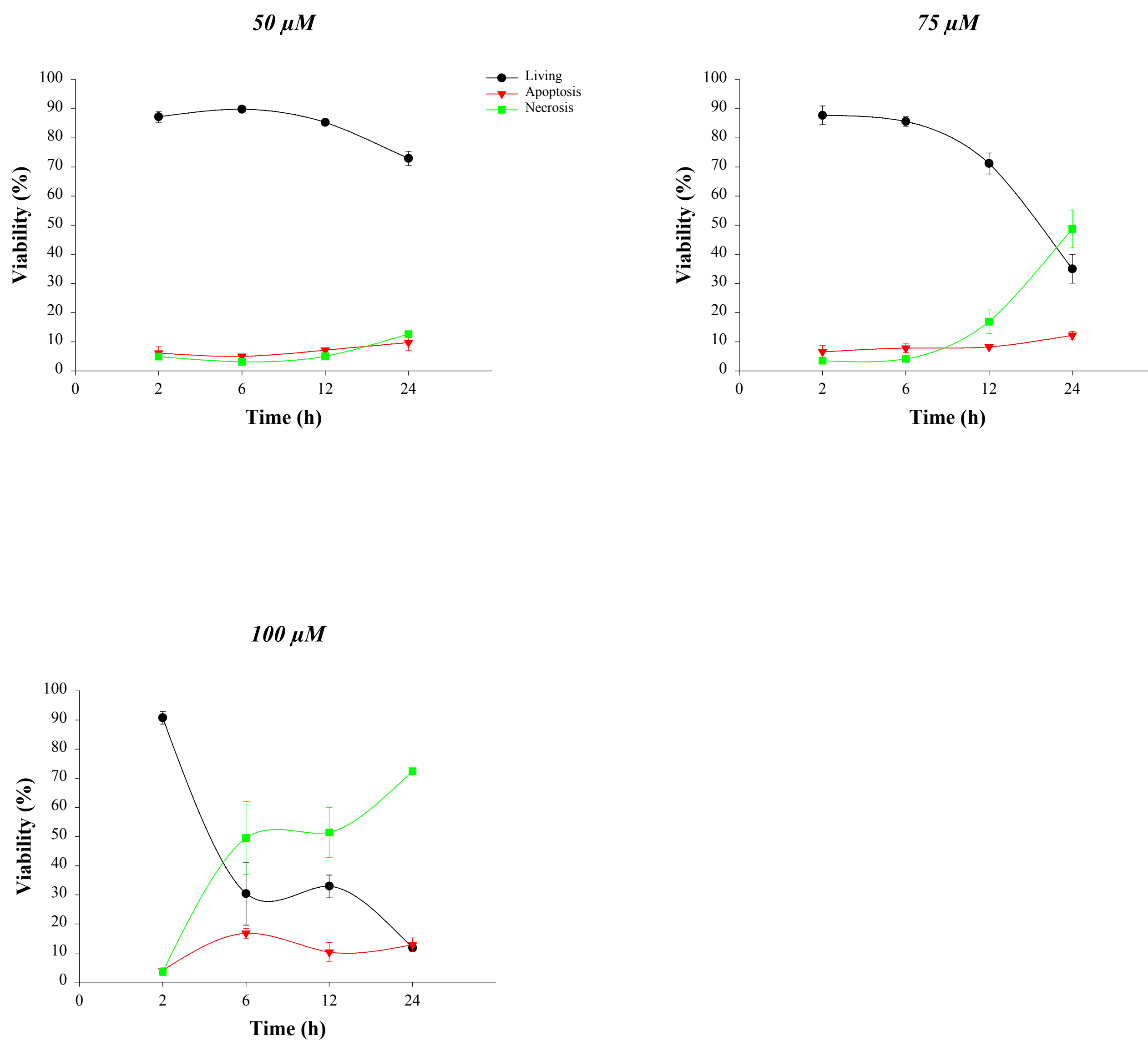
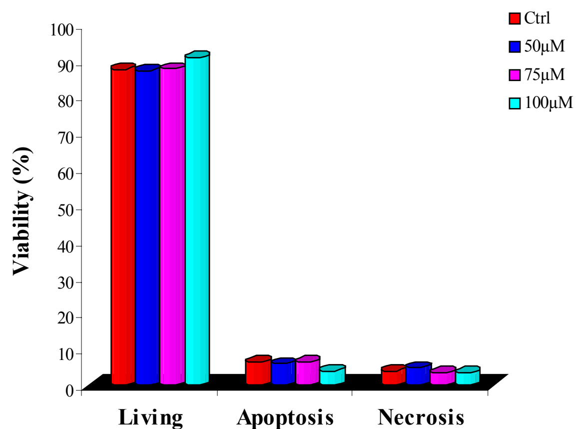
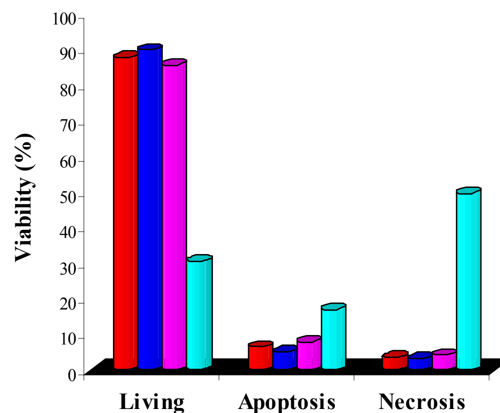


Figure 16.10: Histograms of living, apoptosis and necrosis in Balb/3T3 cells exposed to $(NH_4)_2PtCl_6$

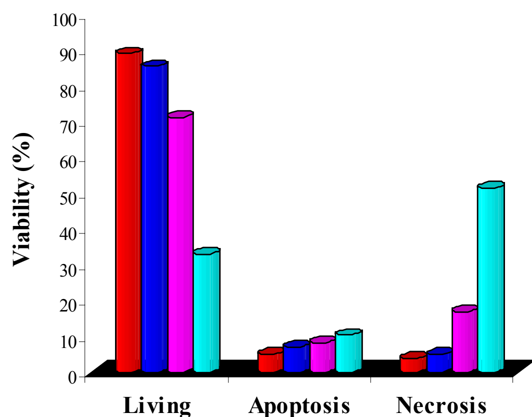
2 hours



6 hours



12 hours



24 hours

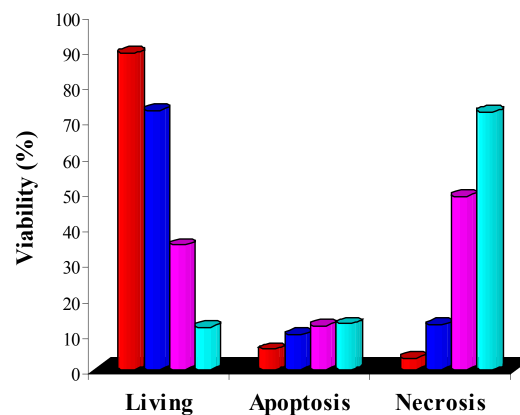


Figure 16.11: Curves of living, apoptosis and necrosis in Balb/3T3 cells exposed to $PtCl_4$

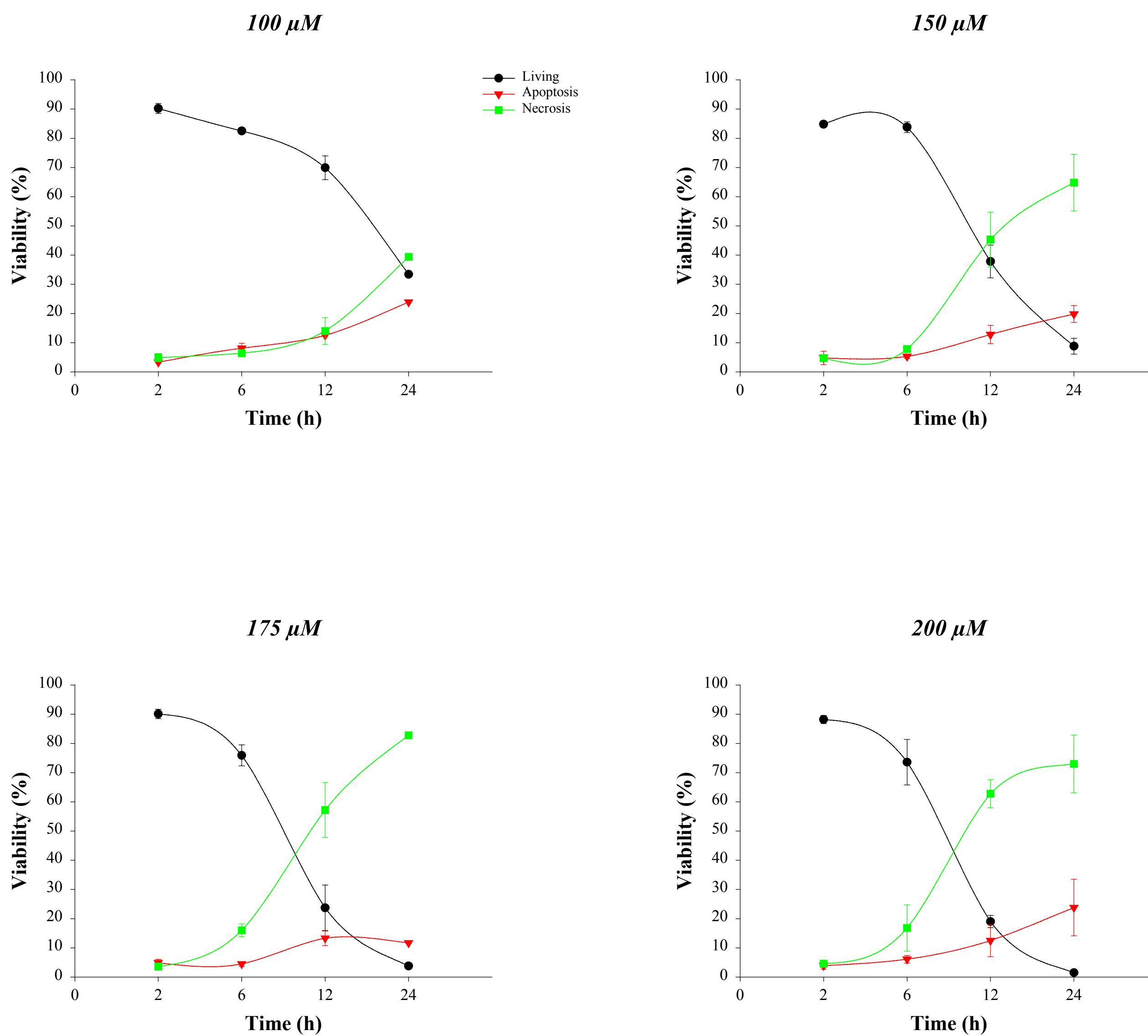
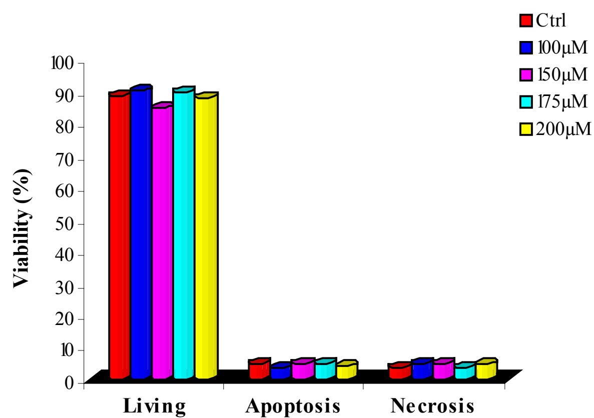
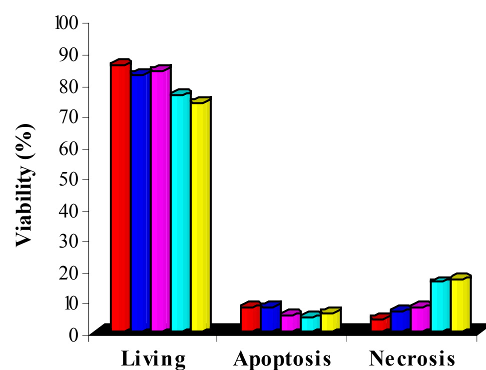


Figure 16.12: Histograms of living, apoptosis and necrosis in Balb/3T3 cells exposed to $PtCl_4$

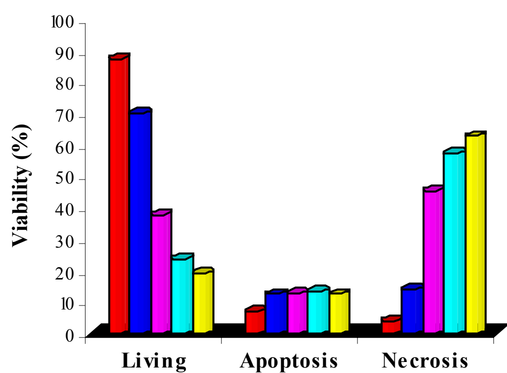
2 hours



6 hours



12 hours



24 hours

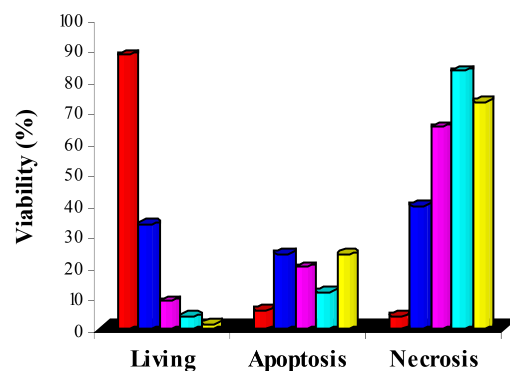


Figure 16.13: Curves of living, apoptosis and necrosis in Balb/3T3 cells exposed to $PtCl_2$

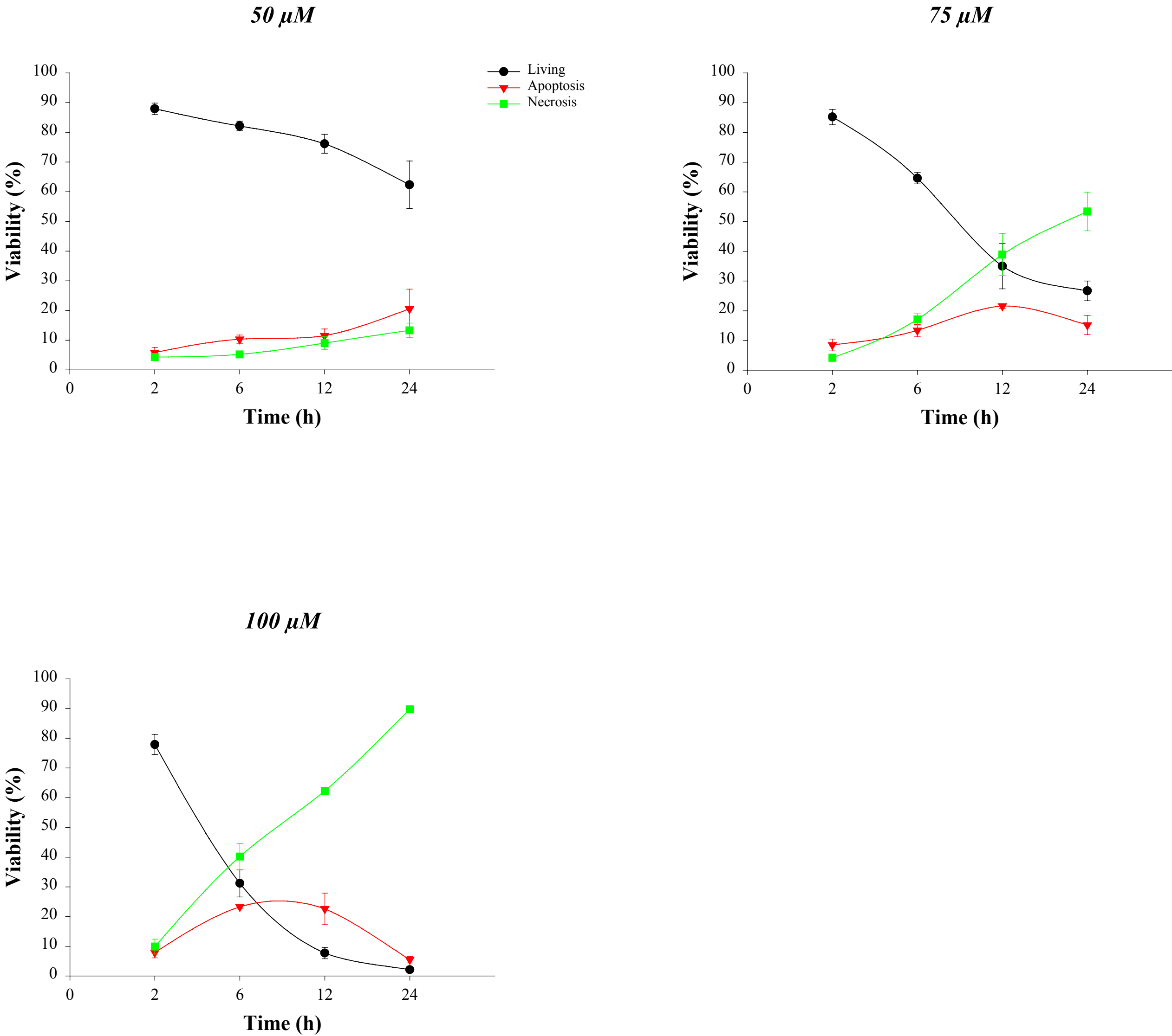
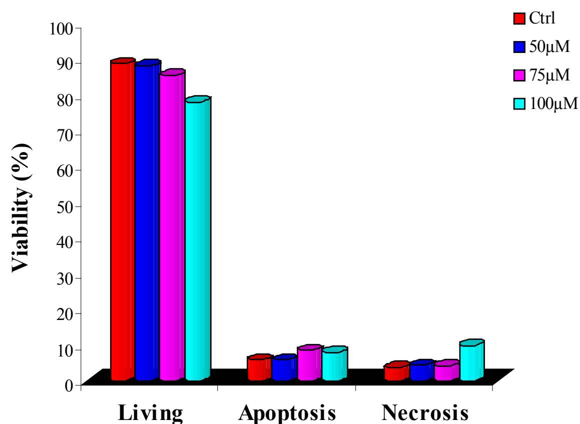
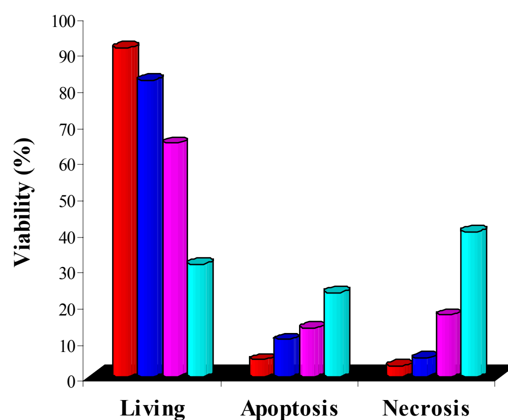


Figure 16.14: Histograms of living, apoptosis and necrosis in Balb/3T3 cells exposed to $PtCl_2$

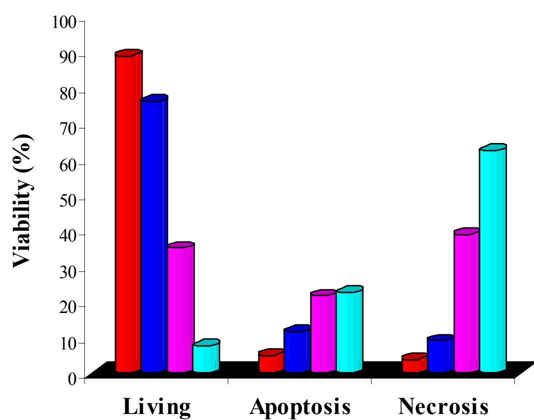
2 hours



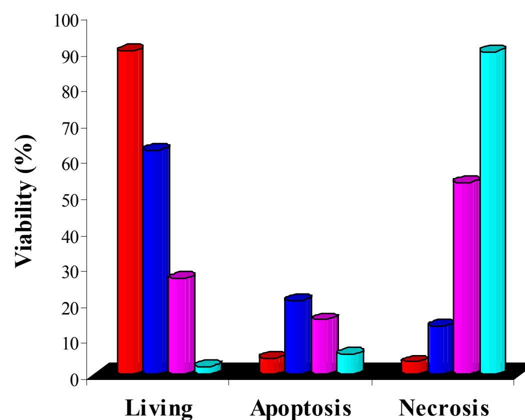
6 hours



12 hours



24 hours



16.2 Measurement of Caspase-3 Activity

Measurement of the enzymatic caspase-3 activity in the Balb/3T3 cells (Section 9.2) was performed to compare the results obtained with the annexin V/PI assay.

Arsenic. Table 16.VI shows an increase of the caspase-3 activity induced by $NaAsO_2$ after 6-hour exposure over all concentrations tested. This confirms the results achieved with the application of the annexin V/PI assay (Figures 16.1-16.2).

However, at 150 μM after 12-hour exposure it was not possible to detect a clear induction of the caspase-3 activity. In fact, as previously explained in Figure 9.3, the scatter related to this treatment showed a high degree of strongly damaged cells.

Table 16.VI: Induction of caspase-3 in Balb/3T3 cells after exposure to $NaAsO_2$

Concentration (μM)	Caspase positive (%)	Caspase negative (%)
	6 h	
0	1.97	97.8
100	7.83	91.5
150	26.2	72.6
175	25.3	73.8
200	38.9	59.6
	12 h	
0	1.32	98.4
150	Not detectable	

Chromium. Exposure to $Na_2CrO_4 \cdot 4H_2O$ showed an increase of caspase-3 activity after 6-hour exposure over the whole range of concentrations tested (Table 16.VII). This confirms apoptotic induction as previously described by the annexin/PI assay (Figures 16.3-16.4).

A clear increase of strongly damaged cells in the total population was evident in the scatter referring to the treatment at 300 µM after 12-hour exposure (see Figure 9.3).

Table 16.VII: Induction of caspase-3 in Balb/3T3 cells after exposure to $Na_2CrO_4 \cdot 4H_2O$

Concentration (µM)	**Caspase positive (%)**	**Caspase negative (%)**
	6 h	
0	1.97	97.8
250	4.78	94.7
300	7.33	91.8
350	15.3	83.9
	12 h	
0	1.32	98.4
300	Not detectable	

Cis-Pt. Table 16.VIII shows no effect induced by *cis*-Pt after 6-hour exposure, whereas an increasing activity of caspase-3 was detected after 12 hours confirming the results obtained with the annexin V/PI assay (Figures 16.5-16.6).

The treatment at 150 μM after 12-hour exposure was hardly detectable. In fact, at the following concentration 200 μM many strongly damaged cells were observed, as revealed by the corresponding scatter (see Figure 9.3).

Table 16.VIII Induction of caspase-3 in Balb/3T3 cells after exposure to cis-Pt

Concentration (μM)	Caspase positive (%)	Caspase negative (%)
	6 h	
0	2.51	97.3
85	1.14	98.7
100	3.53	96.0
	12 h	
0	1.32	98.4
85	26.1	73.0
100	20.1	79.0
150	57.8	39.9
200	Not detectable	

Carbo-Pt. After 6-hour exposure carbo-Pt did not induce caspase-3 activity (Table 16.IX). However, clear evidence for an increased cellular expression of this enzyme was obtained after 12-hour exposure, confirming the data previously obtained by the annexin V/PI assay (Figures 16.7-16.8).

Table 16.IX: Induction of caspase-3 in Balb/3T3 cells after exposure to carbo-Pt

Concentration (µM)	**Caspase positive (%)**	**Caspase negative (%)**
	6 h	
0	0.88	99.0
1500	1.75	97.8
2000	1.73	98.0
	12 h	
0	0.64	99.2
1250	9.89	89.2
1500	13.9	85.2
1750	26.3	72.4
2000	55.1	44.4

(NH$_4$)$_2$PtCl$_6$. The analysis of (NH$_4$)$_2$PtCl$_6$ did not confirm entirely the results obtained by the annexin V/PI assay (Figures 16.9-16.10). This was particularly evident at 100 µM after 6-hour exposure (13.9% of caspase-3 activity), while at 75 µM after 12-hour exposure a further response of the enzyme activity (5.25% of induction) was identified (Table 16.X).

However, as revealed by the corresponding scatters, higher concentrations and longer exposures (100 µM and 75 µM after 12- and 24-hour exposure, respectively) were not detectable due to a large population of strongly damaged cells (see Figure 9.3).

Table 16.X: Induction of caspase-3 in Balb/3T3 cells after exposure to (NH$_4$)$_2$PtCl$_6$

Concentration (µM)	Caspase positive (%)	Caspase negative (%)
	6 h	
0	1.17	98.6
75	2.49	97.3
100	13.9	84.6
	12 h	
0	0.98	98.7
75	5.25	94.1
100	Not detectable	
	24 h	
0	0.98	98.7
75	Not detectable	

PtCl$_4$. Table 16.XI shows that $PtCl_4$ at 150 µM after 12-hour exposure induced caspase-3 activity, unlike the corresponding results obtained by the annexin V/PI assay, which showed an increase of necrosis (Figures 16.11-16.12).

At 200 µM after 6- and 12-hour exposure as well as at 150 µM after 24-hour exposure, a predominant amount of strongly damaged cells within the total population was observed (see Figure 9.3).

Table 16.XI: Induction of caspase-3 in Balb/3T3 cells after exposure to $PtCl_4$

Concentration (µM)	**Caspase positive (%)**	**Caspase negative (%)**
	6 h	
0	1.11	98.6
150	3.12	96.4
200	Not detectable	
	12 h	
0	1.16	98.6
150	25.5	72.9
200	Not detectable	
	24 h	
0	1.99	97.5
150	Not detectable	

<u>$PtCl_2$.</u> Table 16.XII shows an increase of caspase-3 activity induced by 75 μM of $PtCl_2$ after 6- and 12-hour exposure (7% and 14.5% of induction, respectively).

Further experiments at higher concentrations and longer exposure times were not feasible because of a very large amount of cellular debris.

However, at 75 μM after 6- and 12-hour exposure the annexin V/PI assay showed necrosis value higher than apoptosis (Figures 16.13-16.14).

Table 16.XII: Induction of caspase-3 in Balb/3T3 cells after exposure to $PtCl_2$

Concentration (μM)	**Caspase positive (%)**	**Caspase negative (%)**
	6 h	
0	0.85	98.9
75	7.0	92.5
100	Not detectable	
	12 h	
0	1.09	98.6
75	14.5	84.6
100	Not detectable	
	24 h	
0	1.99	97.5
75	Not detectable	

16.3 Nuclear DNA Fragmentation Assay

The present section reports the results of the morphological study based on the detection of apoptosis-induced nuclear DNA fragmentation via fluorescence assay by confocal microscopy (Section 9.3).

Photos 16.1-16.26 refer to the Balb/3T3 cells exposed to $NaAsO_2$, $Na_2CrO_4 \cdot 4H_2O$, *cis*-Pt, carbo-Pt, $(NH_4)_2PtCl_6$, $PtCl_4$, and $PtCl_2$ at different concentrations and exposure times selected on the basis of the results obtained from previous apoptotic studies carried out by the annexin V/PI and the induction of caspase-3 activity assays (Sections 16.1 and 16.2).

Photo 16.1 (negative control):

- All nuclei appeared red, in large amount, with a good shape. This indicates the absence of suffering cells and in particular no DNA fragmentation because of the lack of any green stain (Photo 16.1*a'*).
- The image of cytospin (Photo 16.1*b*) confirms the good conditions of the cells attached in the chamber slide. At the end of the treatment few cells were in suspension with a basal level of apoptosis (see the green staining).

Photo 16.2 (positive control):

- The green staining of the nuclei was very strong (Photo 16.2*a'*). Where DNA fragmentation was more extensive, the green fluorescence increased and the combination of the two stains were more intense.
- The very few cells found with the cytospin (Photo 16.2*b*) confirm that no other factors acted on this positive control except for the treatment with the DNase I enzyme.

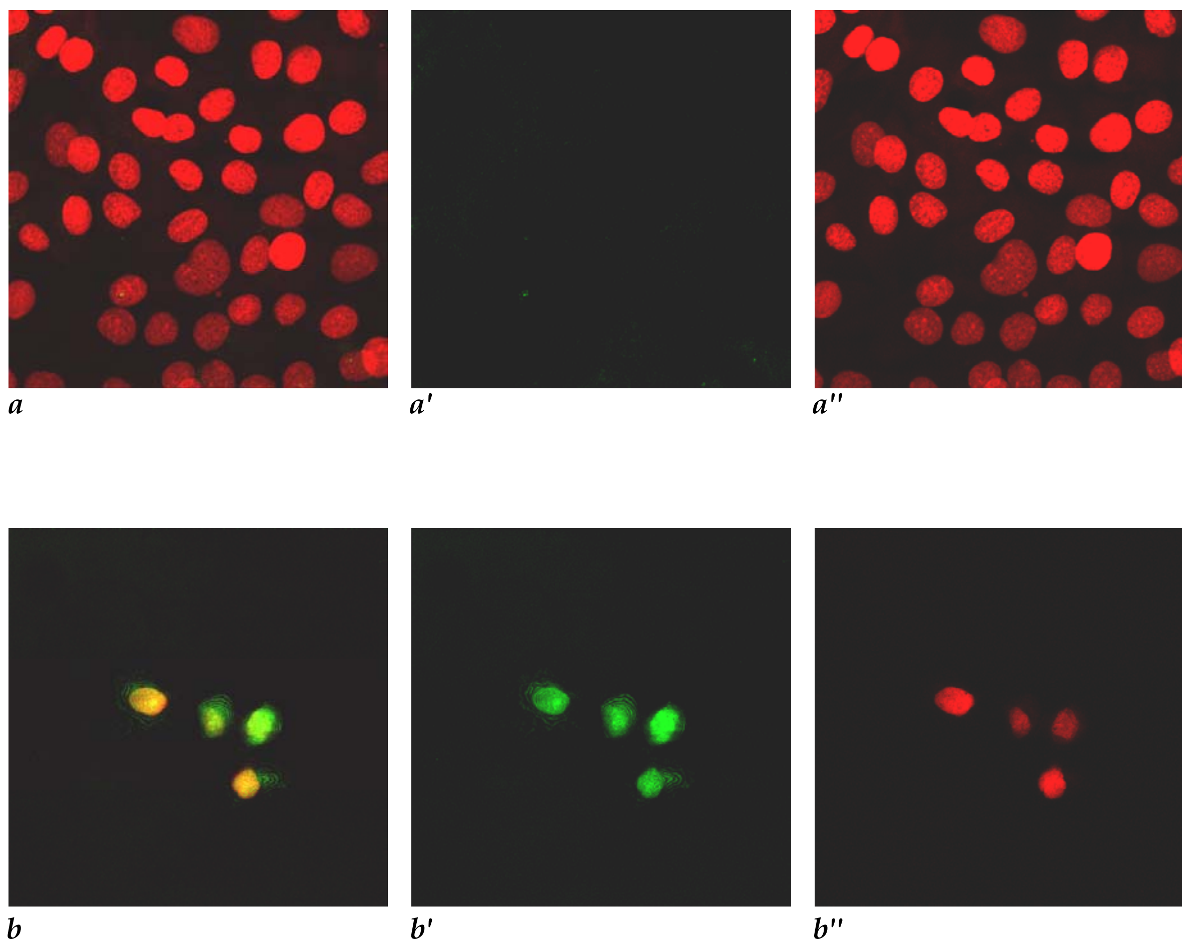


Photo 16.1: DNA fragmentation in Balb/3T3 cells: negative control

Note: *for each sample the series of small letters a (a, a', a'') represents images derived from cells still attached on the bottom of the chamber slide, while the series of small letters b (b, b', b'') refers to cells in suspension and then spotted on slides by cytospin. For a same image the confocal microscope allows three photographs to be taken concurrently.*

The first photograph is obtained using both filters together, each of them specific for one of the two fluorochromes used for the assay, namely, FITC and PI (see photographs a or b). In this case the corresponding stainings appear overlapped.

The second photograph depicts the nuclei eventually stained in green by the FITC fluorochrome (see photographs a' or b').

The third photograph shows red nuclei because of the PI staining (see photographs a'' or b'').

Only where more detailed explanation is necessary, all three images are shown, instead of a single photograph with the combined filters.

With regard to Photos 16.1b, 16.2 and 16.3: ├───┤ *= 52.2 μm.*

With regard to Photos 16.1a, 16.4-16.26: ├───┤ *= 31.2 μm.*

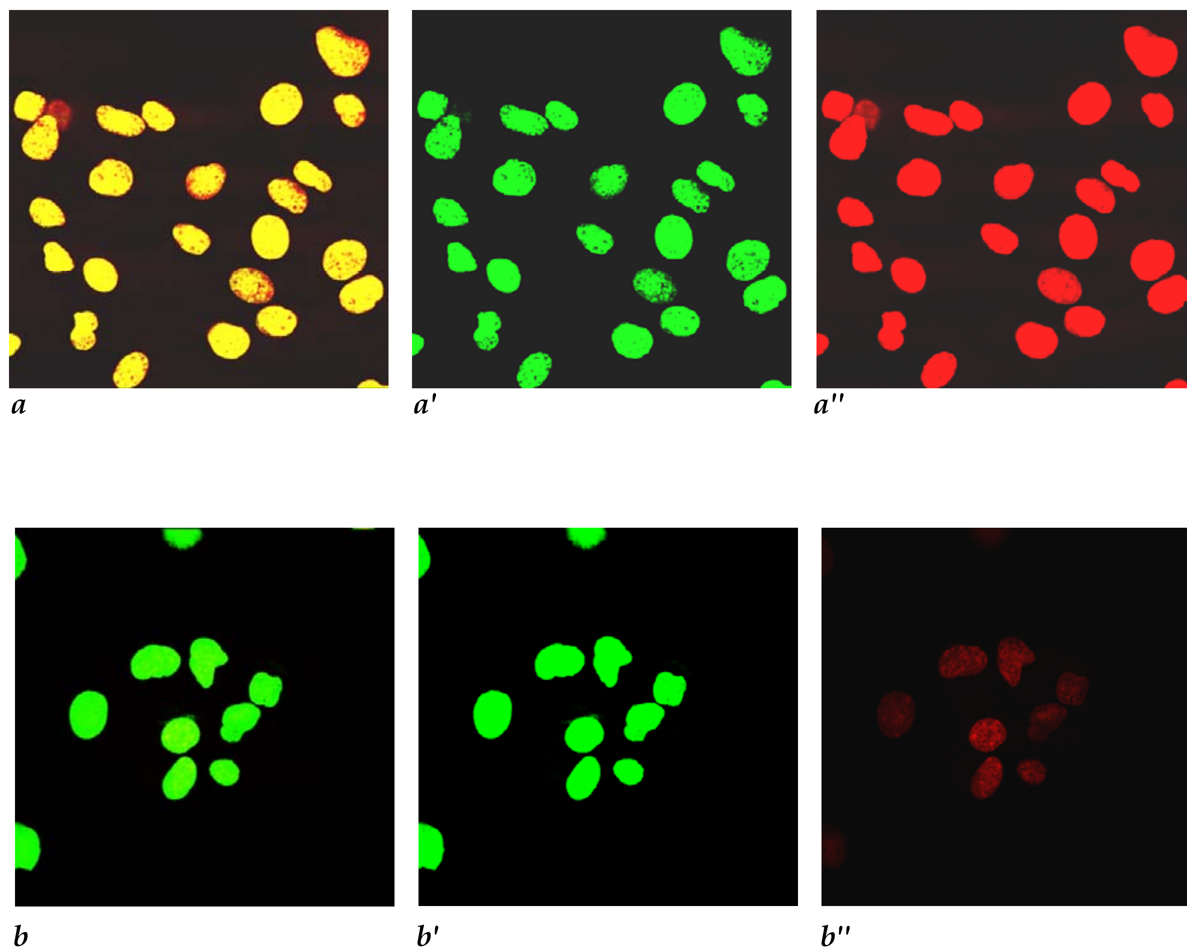


Figure 16.2: DNA fragmentation in Balb/3T3 cells: positive control

Photos 16.3-16-5 (Arsenic): $NaAsO_2$ was analysed at concentrations 100 µM (6-hour exposure) as well as 150 µM (6- and 12-hour exposure).

Photo 16.3: after 6-hour exposure to 100 µM the DNA did not appear particularly fragmented.

Photo 16.4: at 150 µM the cells (few after the treatment) contained nuclei well stained by fluorescein (Photo 16.3*a*). In the cytospin image a more irregular shape of the nuclei is evident (Photo 16.3*b*). This treatment confirms an increase of apoptosis after 6-hour exposure, as already observed in studies with annexin V/PI (Figures 16.1-16.2) and caspase-3 activity (Table 16.VI).

Photo 16.5: at 150 µM after 12-hour exposure no cells were scored in the chamber slide (photograph not shown). Thus, this exposure period represents an extreme condition further confirmed by the corresponding cytospin, where the cells were very few; their nuclei appeared condensed and necrotic.

Photo 16.3: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 100 µM $NaAsO_2$

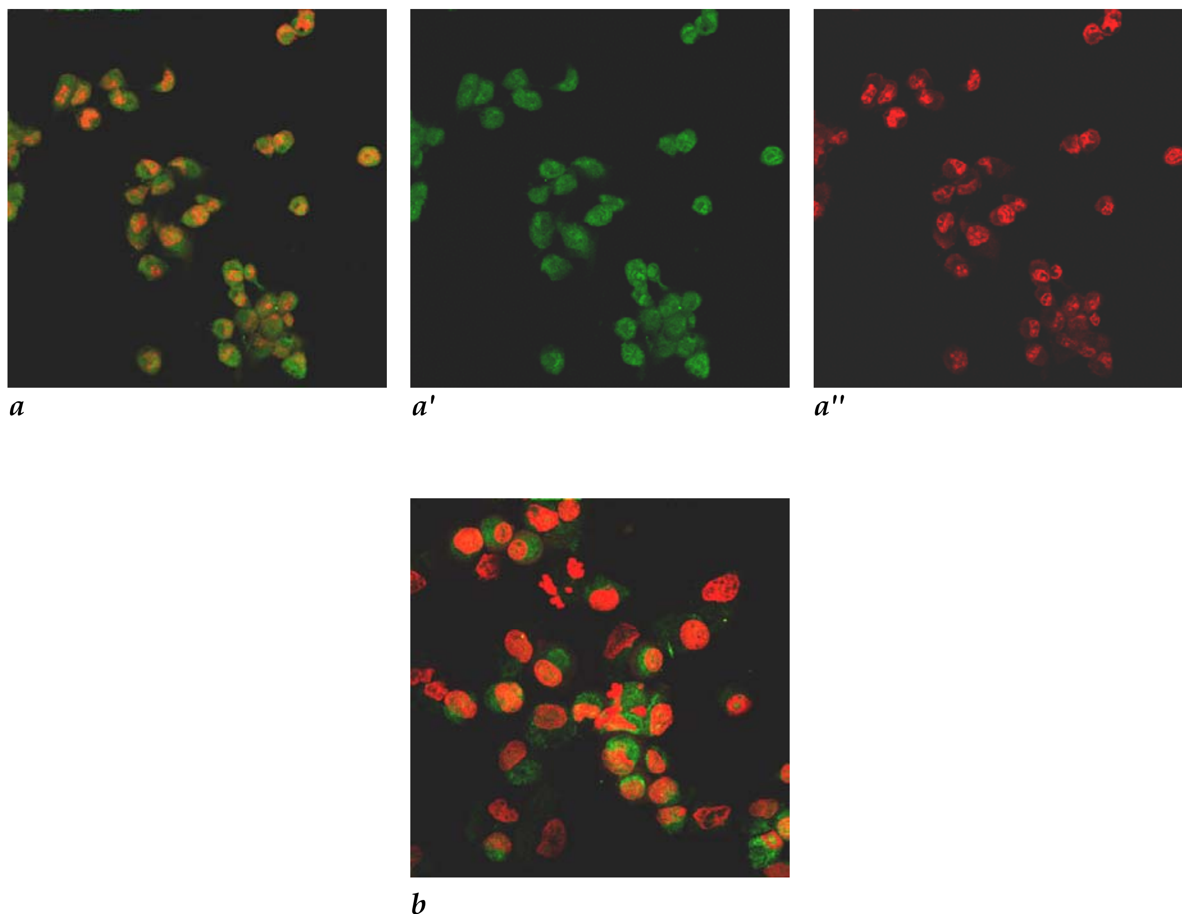
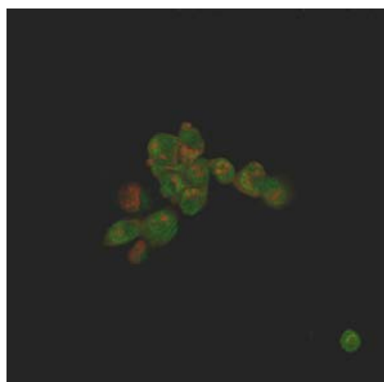
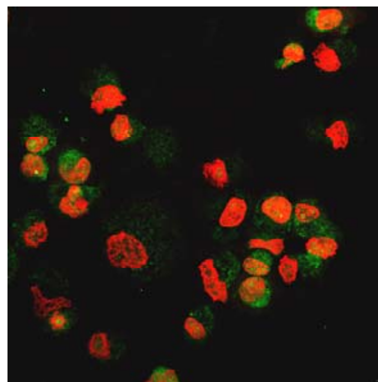


Photo 16.4: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 150 µM $NaAsO_2$

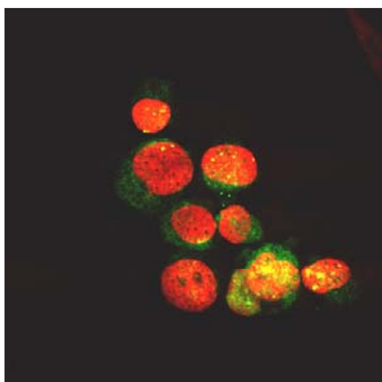


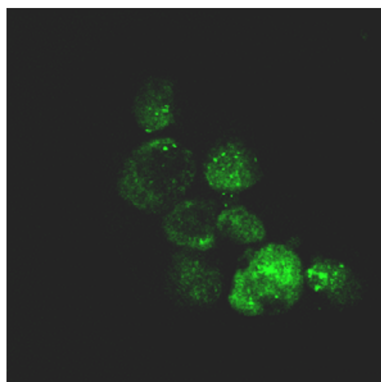
a

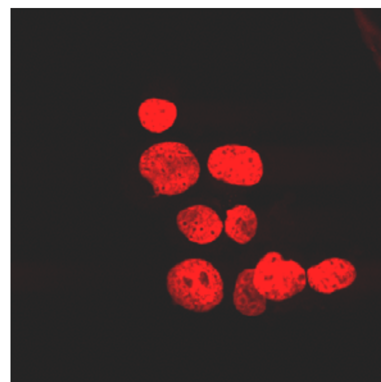


b

Photo 16.5: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 150 µM $NaAsO_2$ (Images of cytospin only)





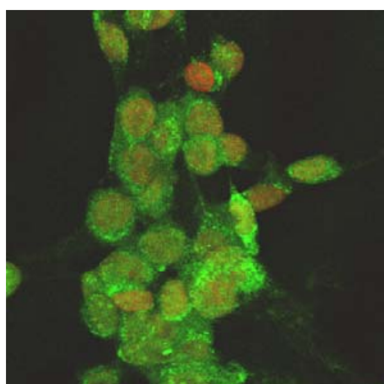


Photos 16.6-16-8 (Chromium): $Na_2CrO_4 \cdot 4H_2O$ was analysed at concentrations 250 µM (6-hour exposure) as well as 300 µM (6- and 12-hour exposure).

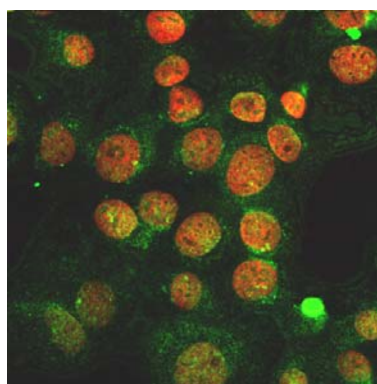
Photo 16.6-16.7: at 250 and 300 µM after 6-hour exposure an induction of apoptosis was particular evident, as shown by the intense FITC staining of the nuclei. This confirms previous data obtained by annexin V/PI (Figures 16.3-16.4) and caspase-3 assay (Table 16.VII), where Cr(VI) was able to induce apoptosis only after 6-hour exposure.

Photo 16.8: at 300 µM after 12-hour exposure a large amount of DNA fragmentation in the cytospun cells was observed (Photo 16.8*b*). This represents a new finding in contrast with previous results of the annexin/PI and the caspase-3 assays that detected strongly damaged cells.

Photo 16.6: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 250 µM $Na_2CrO_4 \cdot 4H_2O$

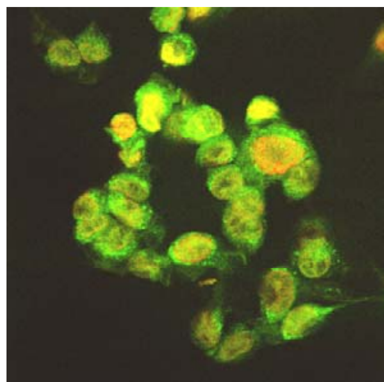


a

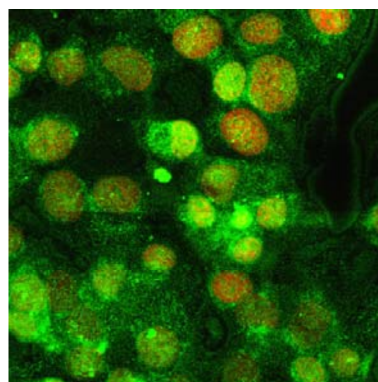


b

Photo 16.7: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 300 µM $Na_2CrO_4 \cdot 4H_2O$

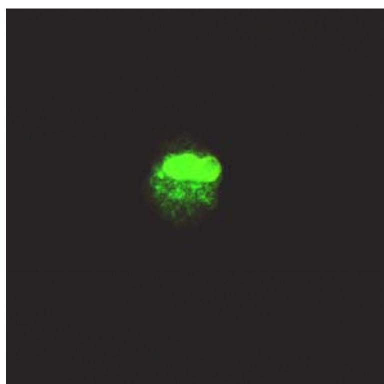


a

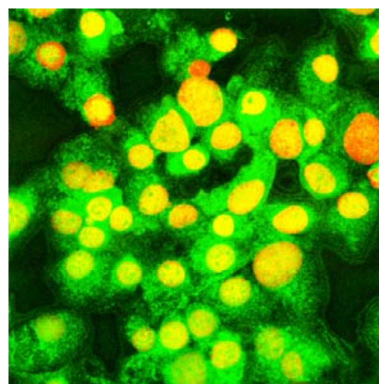


b

Photo 16.8: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 300 µM $Na_2CrO_4 \cdot 4H_2O$



a



b

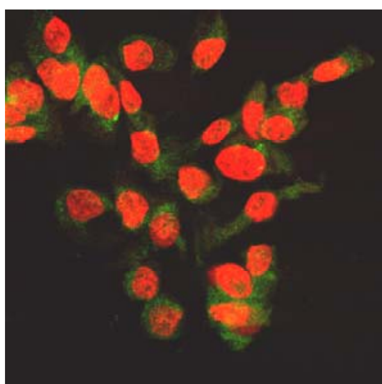
Photos 16.9-16-11 (<u>cis-Pt</u>): *cis*-Pt was analysed at concentrations 100 μM (6-hour exposure) as well as 85 and 200 μM (12-hour exposure).

Photo 16.9: at 100 μM after 6-hour exposure there was no DNA fragmentation.

Photo 16.10: at 85 μM after 12-hour exposure a very strong green fluorescence in the nuclei was evident. This confirms previous data as obtained by the annexin V/PI (Figures 16.5-16.6) and the induction of caspase-3 activity (Table 16.VIII) assays, which showed *cis*-Pt as inducer of apoptosis particularly after 12-hour exposure.

Photo 16.11: at 200 μM after 12-hour exposure a necrotic process was evident especially in the cytospun cells (Photo 16.11*b*).

Photo 16.9: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 100 μM cis-Pt



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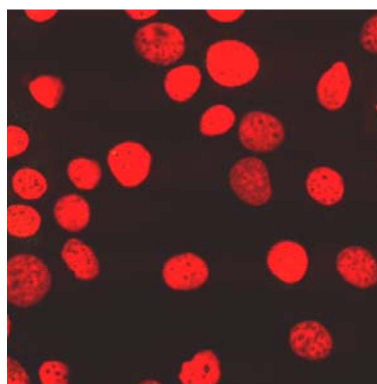
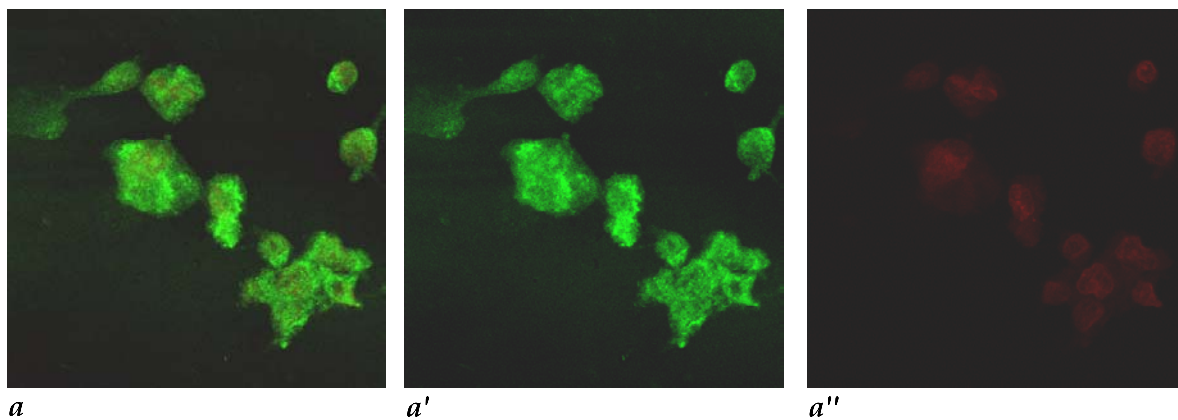
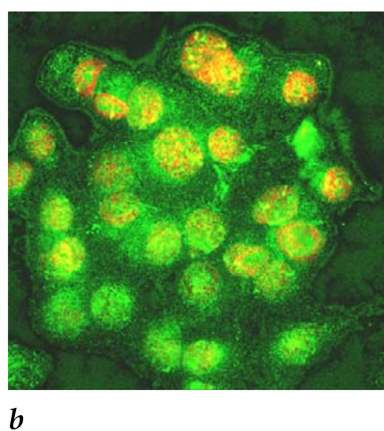


b

Photo 16.10: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 85 µM cis-Pt

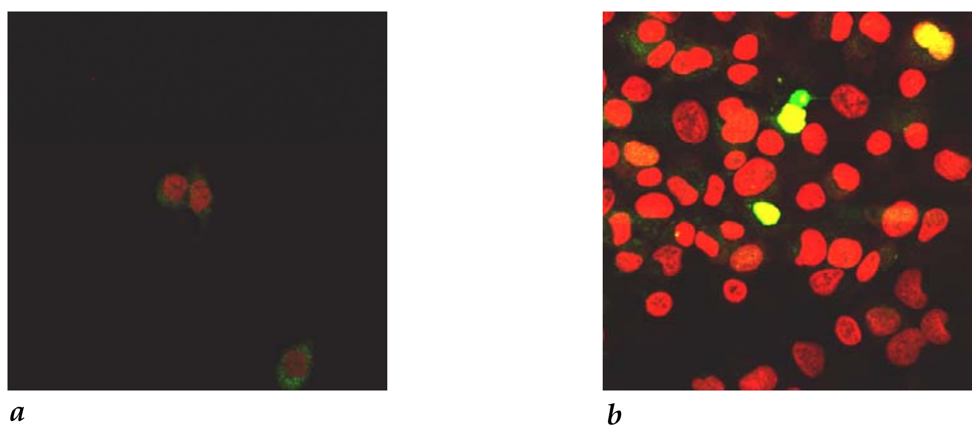


a ***a'*** ***a''***



b

Photo 16.11: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 200 µM cis-Pt



a ***b***

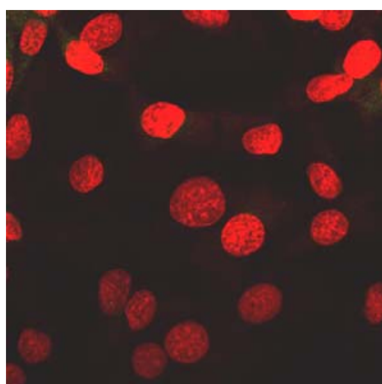
Photos 16.12-16-14 (carbo-Pt): carbo-Pt was analysed at concentrations 1750 μM (6-hour exposure) as well as 1500 and 2000 μM (12-hour exposure).

Photo 16.12: at 1750 μM after 6-hour exposure the cells appeared normal.

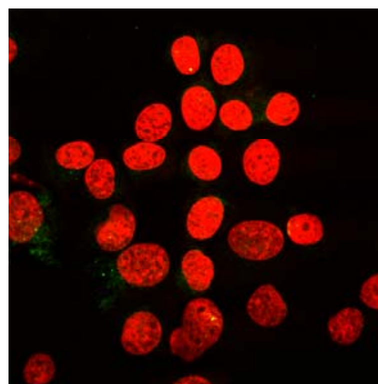
Photo 16.13: at 1500 μM after 12-hour exposure most of cells in chamber slide was detached. However, the cytospun cells showed condensed chromatin at the nucleus periphery and the FITC staining increased (Photo 16.13*b*). This data confirm previous results obtained by annexin V/PI (Figures 16.7-16.8) and caspase-3 activity (Table 16.IX) assays showing carbo-Pt as inducer of apoptosis after 12-hour exposure.

Photo 16.14: less fluorescence and altered nucleus shape revealed suffering cells that were probably very strongly damaged by this treatment.

Photo 16.12: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 1750 μM carbo-Pt

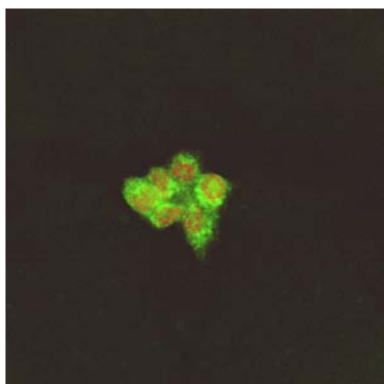


a

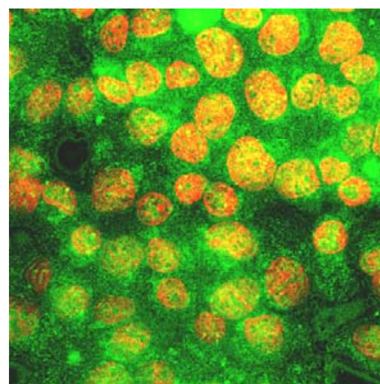


b

Photo 16.13: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 1500 µM carbo-Pt

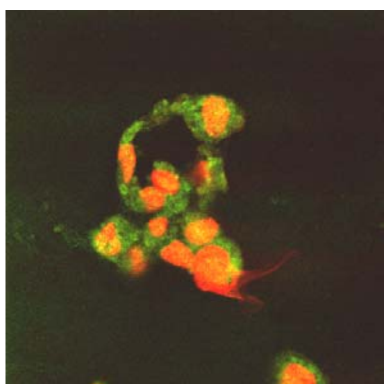


a



b

Photo 16.14: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 2000 µM carbo-Pt



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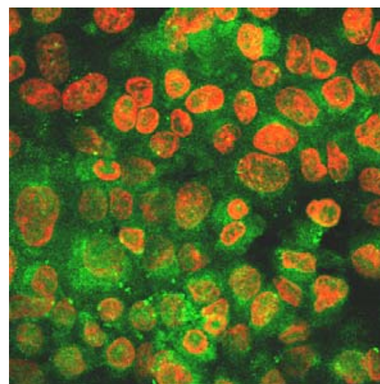
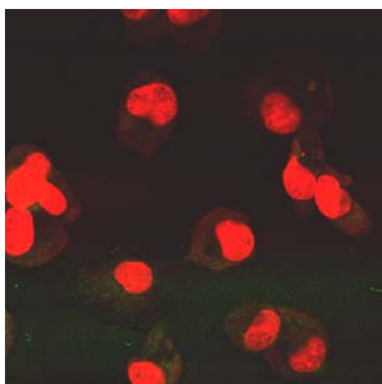


b

Photos 16.15-16-18 (<u>(NH$_4$)$_2$PtCl$_6$</u>): $(NH_4)_2PtCl_6$ was analysed at concentrations 75 and 100 µM (6- and 12-hour exposure).

Photo 16.15-16.18: as confirmation of the results obtained by the annexin V/PI assay (Figures 16.9-16.10) and partly by the induction of caspase-3 activity (Table 16.X), the treatments reported by the following photographs did not identify nuclear fragmentation at any concentration tested and after any exposure times considered. In particular, Photos 16.17 and 16.18 depict altered nucleus shape and decreased cell number.

Photo 16.15: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 75 µM $(NH_4)_2PtCl_6$



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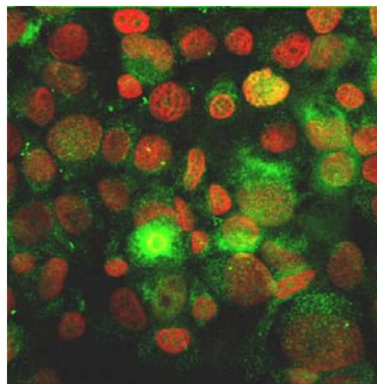
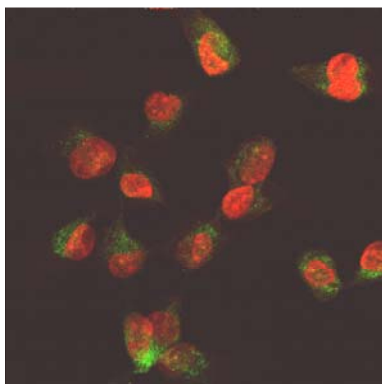
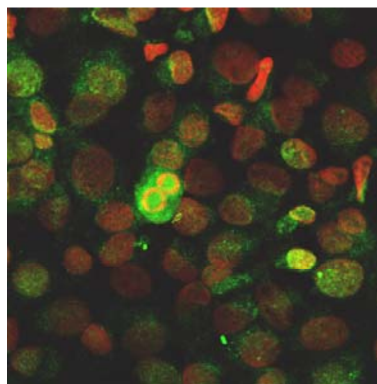


b

Photo 16.16: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 100 µM $(NH_4)_2PtCl_6$

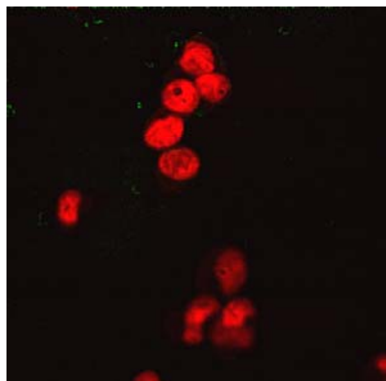


a



b

Photo 16.17: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 75 µM $(NH_4)_2PtCl_6$



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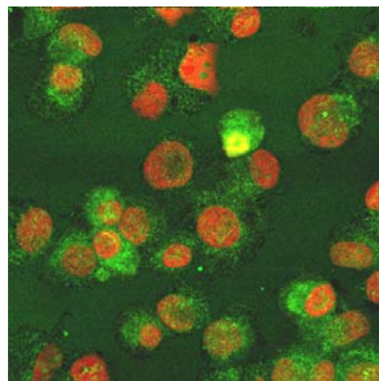
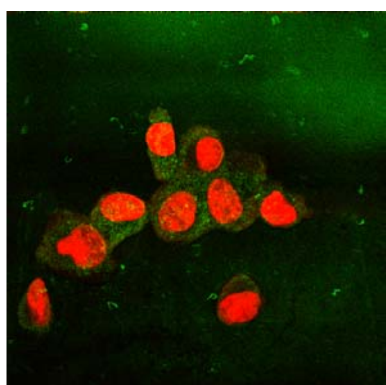
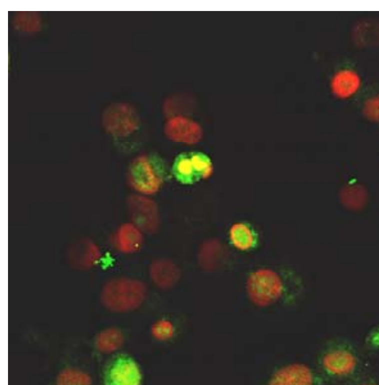


b

Photo 16.18: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 100 µM $(NH_4)_2PtCl_6$



a



b

Photos 16.19-16-22 (<u>$PtCl_4$</u>): $PtCl_4$ was analysed at concentrations 100 and 150 µM (6- and 12-hour exposure).

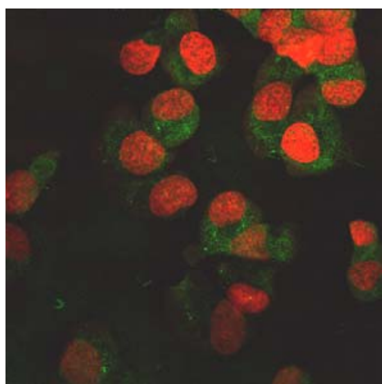
Photo 16.19: at 100 µM after 6-hour exposure most of cytospun cells showed a small, even clear, level of DNA fragmentation (Photo 16.19*b*). This is not in agreement with the data obtained by annexin V/PI (Figures 16.11-16.12) and caspase-3 activity (Table 16.XI), where no apoptosis was found after 6-hour exposure.

Photo 16.20: at 150 µM after 6-hour exposure no DNA fragmentation was detected. The cells in the chamber slide showed altered nucleus shape (Photo 16.20*a*).

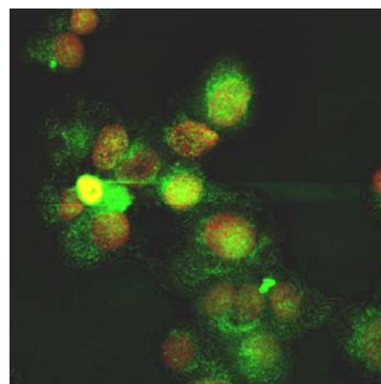
Photo 16.21: at 100 µM after 12-hour exposure the cells did not appear fragmented.

Photo 16.22: at 150 µM after 12-hour exposure most of cells were present in the cytospin (Photo 16.22*b*). They showed nuclei intensively stained by FITC with chromatin often condensed at the nucleus periphery. This confirms the induction of caspase-3 activity as reported in Table 16.XI.

Photo 16.19: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 100 µM $PtCl_4$

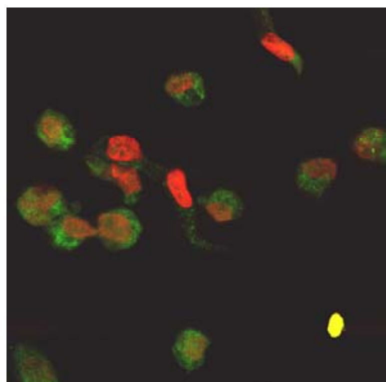


a

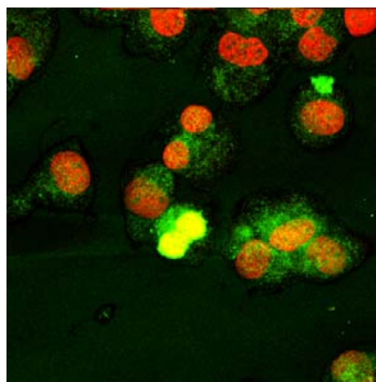


b

Photo 16.20: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 150 µM $PtCl_4$

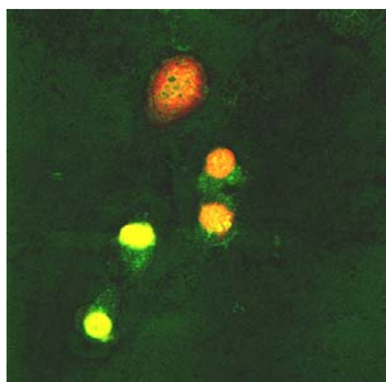


a

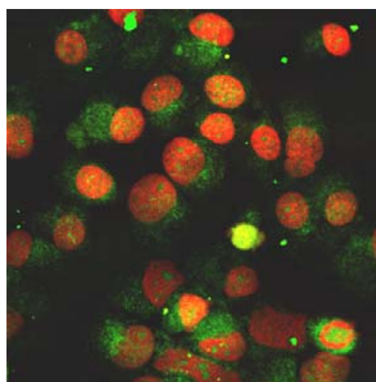


b

Photo 16.21: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 100 µM $PtCl_4$

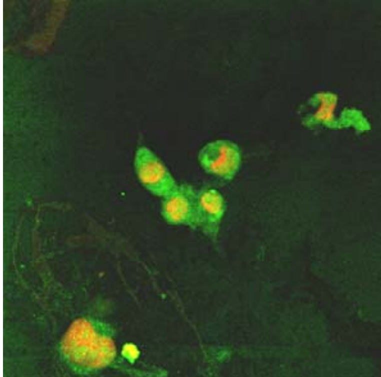


a

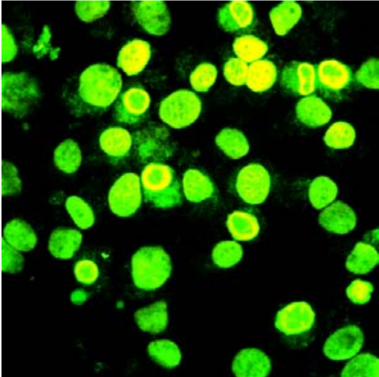


b

Photo 16.22: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 150 μM $PtCl_4$



a



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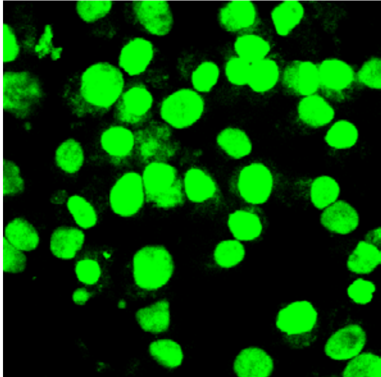
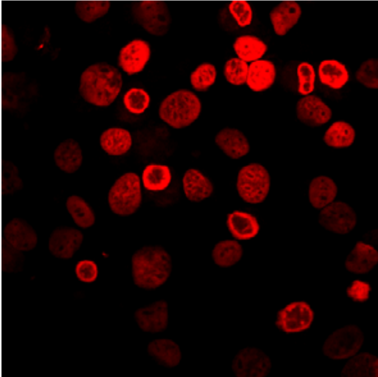


b'



b''

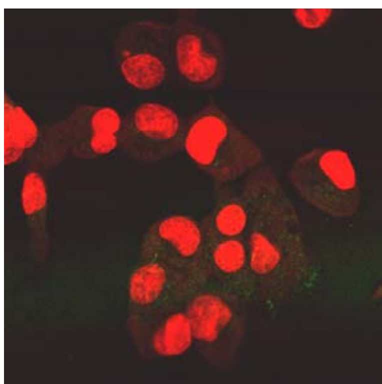
Photos 16.23-16-26 (<u>$PtCl_2$</u>): $PtCl_2$ was analysed at concentrations 75 and 100 μM (6- and 12-hour exposure).

Photo 16.23-16.24: at 75 and 100 μM after 6-hour exposure there was no evidence of DNA fragmentation both in the chamber slide and in the cytospin. These treatments confirm the lack of apoptosis induction after 6-hour exposure detected by annexin V/PI (Figures 16.13-16.14), although at 75 μM Table 16.XII shows 7% of caspase-3 activity.

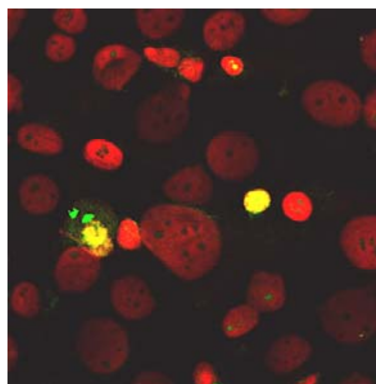
Photo 16.25: at 75 μM after 12-hour exposure the nuclei appeared little more stained by fluorescein, particularly for the few cells remained in the chamber slide (Photo 16.25*a*). This is not in agreement with the induction of caspase-3 activity reported in Table 16.XII.

Photo 16.26: at 100 μM after 12-hour exposure no cells were found in the chamber slide (photograph not shown). The corresponding cytospun cells appeared strongly damaged.

Photo 16.23: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 75 μM $PtCl_2$

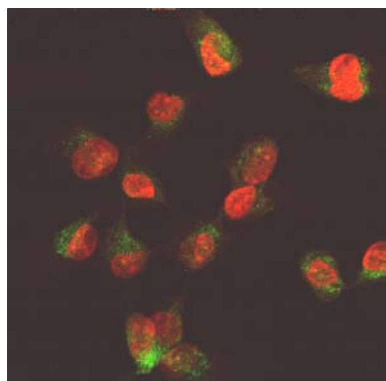


a

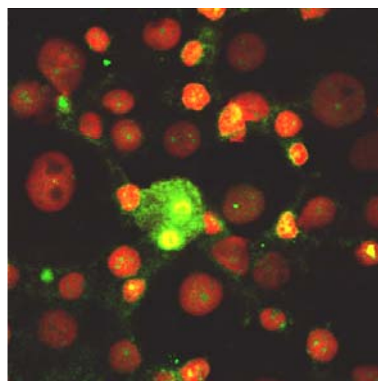


b

Photo 16.24: DNA fragmentation in Balb/3T3 cells after 6-hour exposure to 100 µM $PtCl_2$

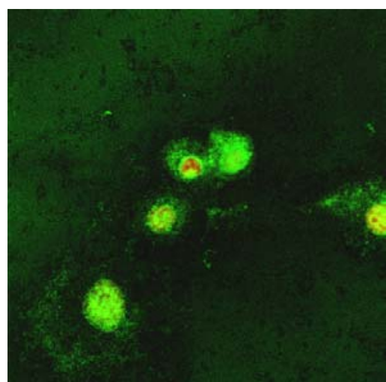


a

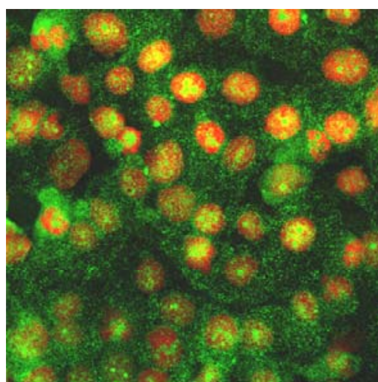


b

Photo 16.25: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 75 µM $PtCl_2$

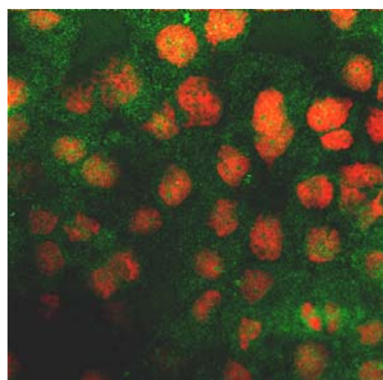


a



b

Photo 16.26: DNA fragmentation in Balb/3T3 cells after 12-hour exposure to 100 µM $PtCl_2$ (Image of cytospin only)



16.4 Hoechst 33342 / PI Assay

Photos 16.27-16.34 show the results of the morphological study on $NaAsO_2$, $Na_2CrO_4 \cdot 4H_2O$, *cis*-Pt, carbo-Pt, $(NH_4)_2PtCl_6$, $PtCl_4$ and $PtCl_2$ concerning the identification of compacted state of chromatin as marker for apoptosis. The investigation was carried out applying the Hoechst 33342/PI assay (Section 9.4) on the Balb/3T3 cells via fluorescence microscopy.

Photo 16.27 shows very good conditions of the negative control. The nucleus shape was normal and the Hoechst staining was not brilliant, while few cells appeared red and therefore dead.

Compared to this control, the metal compounds tested can be classified according to the different effects induced in the cells. The experiments carried out testing $NaAsO_2$ (150 µM, 6-hour exposure, Photo 16.28), $Na_2CrO_4 \cdot 4H_2O$ (300 µM, 6-hour exposure, Photo 16.29) and *cis*-Pt (85 µM, 12-hour exposure, Photo 16.30) were characterised by an increase of the Hoechst fluorescence (notably in *cis*-Pt as shown in Photo 16.30*a*). This indicates a condensed state of the chromatin. Moreover, apoptotic bodies were particularly evident for $NaAsO_2$ (see arrows in Photo 16.28*a*) and, to a less extent, also for $Na_2CrO_4 \cdot 4H_2O$ (see arrows in Photo 16.29*a*). In the case of *cis*-Pt fragmented chromatin was observed (see arrows in Photo 16.30*a*).

Carbo-Pt (1500 µM, 12-hour exposure, Photo 16.31) and $PtCl_4$ (100 µM, 12-hour exposure, Photo 16.33) induced chromatin condensation in many cells as revealed by the very bright fluorescence of the Hoechst staining. Few of these cells were found necrotic.

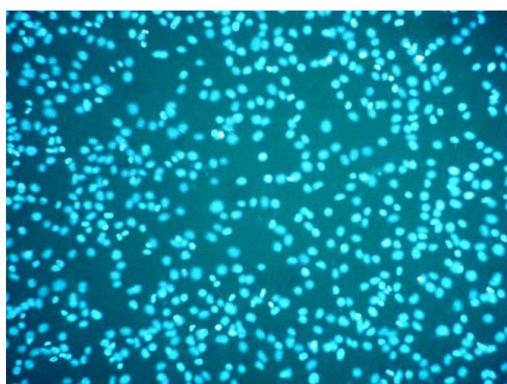
On the contrary, the strong brightness detected in the cells exposed 12 hours to $(NH_4)_2PtCl_6$ (Photo 16.32) and $PtCl_2$ (Photo 16.34) at concentration 75 µM corresponded to a high level of necrosis as confirmed by the PI staining (Photos 16.32*b* and 16.34*b*).

Note: *for each sample one filter was used for the Hoechst dye, while the second filter for the PI dye. As consequence, two individual photographs of each sample were obtained.*

The first photograph depicts the living (blue staining) as well as the supposed apoptotic cells (brilliant blue staining) stained by Hoechst (see photographs a).

The second photograph shows the red PI staining, which identifies dead cells (see photographs b).

Photo 16.27: Chromatin condensation in Balb/3T3 cells: negative control (200X)



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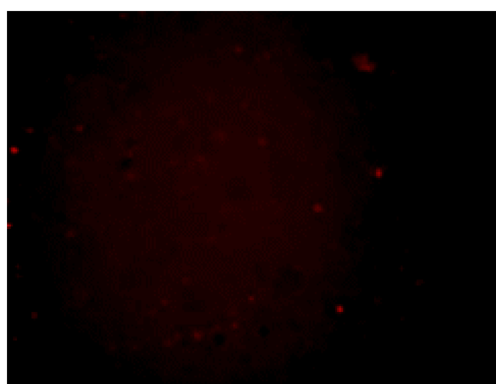
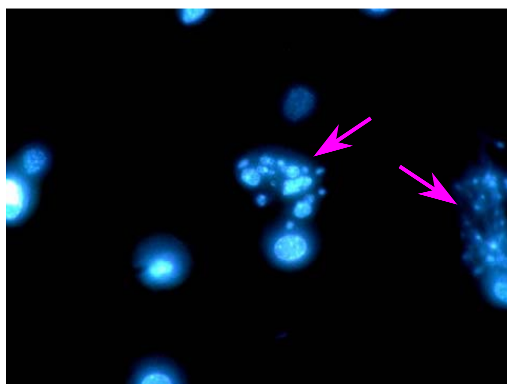
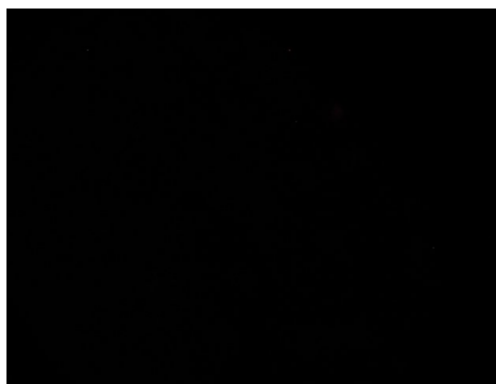


b

Photo 16.28: Chromatin condensation in Balb/3T3 cells after 6-hour exposure to 150 µM $NaAsO_2$ (400X)

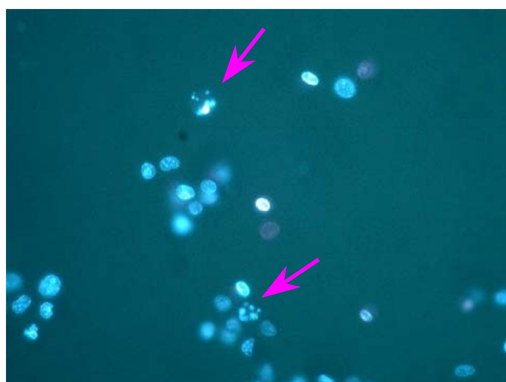


a

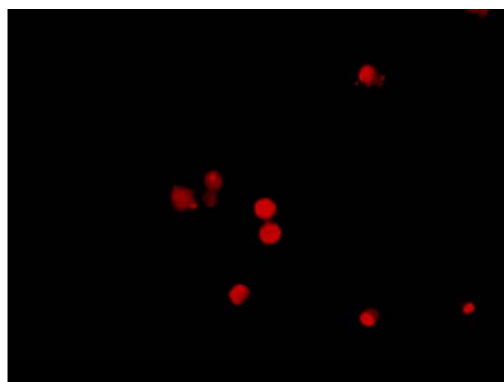


b

Photo 16.29: Chromatin condensation in Balb/3T3 cells after 6-hour exposure to 300 µM $Na_2CrO_4 \cdot 4H_2O$ (200X)

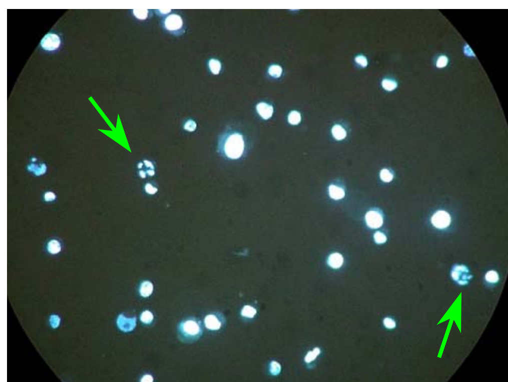


a



b

Photo 16.30: Chromatin condensation in Balb/3T3 cells after 12-hour exposure to 85 µM cis-Pt (200X)



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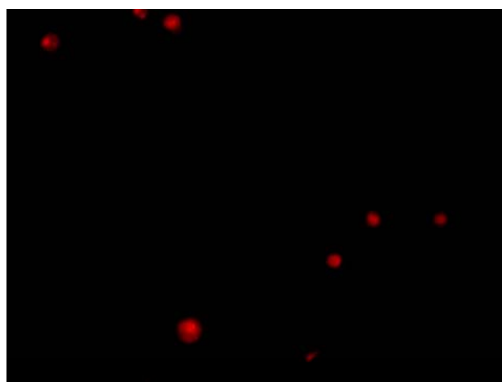
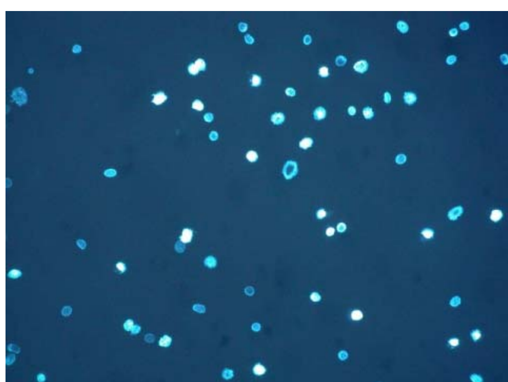


b

Photo 16.31: Chromatin condensation in Balb/3T3 cells after 12-hour exposure to 1500 µM carbo-Pt (150X)



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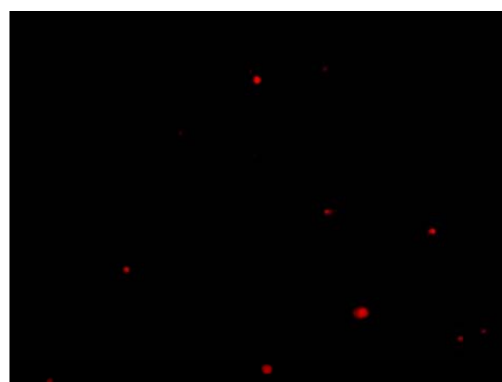
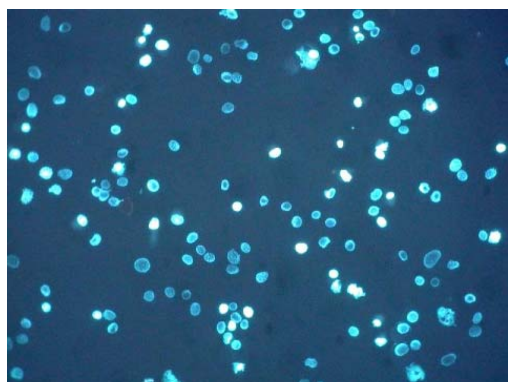
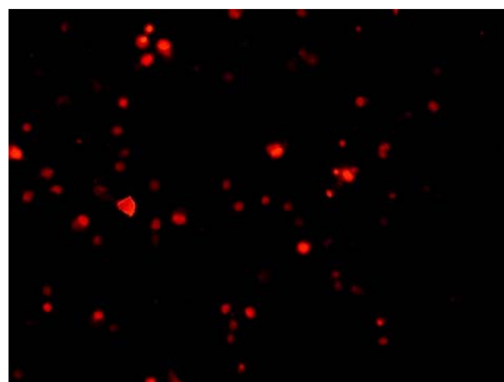


b

Photo 16.32: Chromatin condensation in Balb/3T3 cells after 12-hour exposure to 75 µM $(NH_4)_2PtCl_6$ (150X)

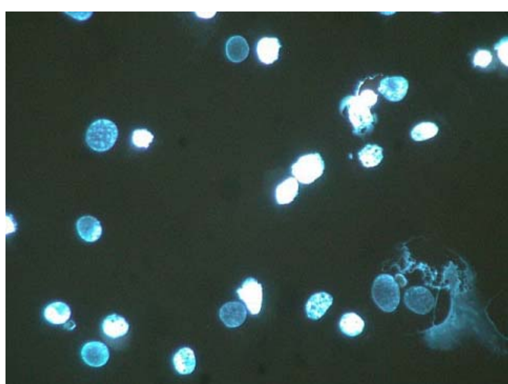


a

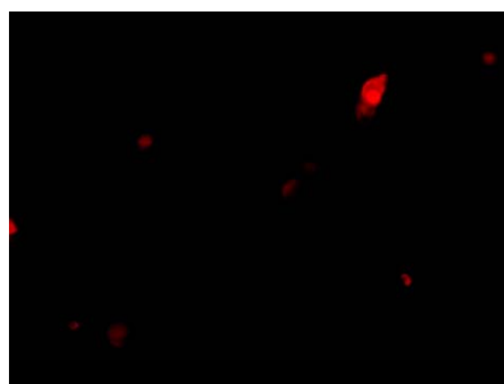


b

Photo 16.33: Chromatin condensation in Balb/3T3 cells after 12-hour exposure to 100 µM $PtCl_4$ (300X)

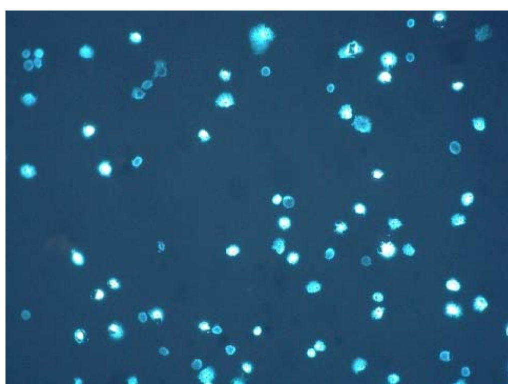


a

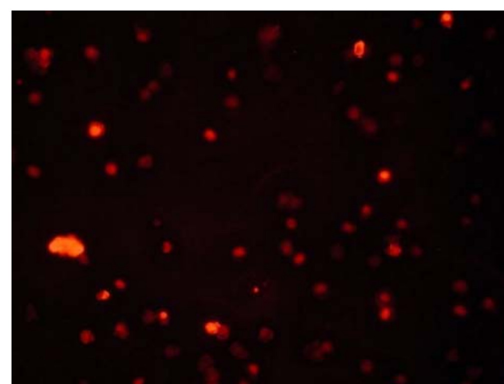


b

Photo 16.34: Chromatin condensation in Balb/3T3 cells after 12-hour exposure to 75 µM $PtCl_2$ (150X)



a



b

16.5 Analytical and Morphological Considerations

The following remarks are reported for each metal compound tested in order to summarise the corresponding results showed in sections 16.1-16.4.

Arsenic. $NaAsO_2$ was positive after 6-hour exposure with 32.6% and 25.3% at 175 µM as induction of apoptosis detected by annexin V/PI and induction of caspase-3 activity assays, respectively (Figures 16.1-16.2, Table 16.VI). Evidence of DNA fragmentation (Photo 16.4) and apoptotic bodies formation (Photo 16.28) was revealed at 150 µM after 6-hour exposure.

Chromium. Induction of apoptosis was confirmed after 6-hour exposure at all concentrations tested of $Na_2CrO_4{\cdot}4H_2O$, whose values varied from 4.78% to 21.2% taking into account the annexin V/PI and the induction of caspase-3 activity assays (Figures 16.3-16.4; Table 16.VII). Nuclear fragmentation (Photo 16.7) and apoptotic bodies formation (Photo 16.29) were identified after 6-hour exposure at 300 µM. Nevertheless, Photo 16.8 shows extensive DNA fragmentation and chromatin condensation particularly at the nucleus periphery at the concentration 300 µM after 12-hour exposure.

Cis-Pt. The results obtained from the experiments concerning *cis*-Pt confirmed the ability of this antitumour agent to induce apoptosis in the Balb/3T3 cell line. Ranging from 85 µM to 150 µM after 12-hour exposure, both cytofluorimetric applications detected values of apoptosis varying from 26.1% to 57.8% (Figures 16.5-16.6; Table 16.VIII). At 85 µM after 12-hour exposure nuclear DNA fragmentation was detected via confocal (Photo 16.10) and fluorescence (Photo 16.30) microscopy.

Carbo-Pt. Induction of apoptosis was evident after 12-hour exposure with values varying from 9.89% to 55.1% as detected by the annexin V/PI and the induction of caspase-3 activity assays (Figures 16.7-16.8; Table 16.IX). In particular, Photos 16.13 and 16.31 indicate that at 1500 µM after 12-hour exposure the cells showed intense chromatin condensation.

$PtCl_4$. Chromatin condensation and DNA fragmentation were observed respectively at 100 µM (Photo 16.33) and 150 µM (Photo 16.22) after 12-hour exposure, while 25.5% of induction of caspase-3 activity was demonstrated at 150 µM

after 12-hour exposure (Table 16.XI). However, no apoptosis was detected by the annexin V/PI assay (Figures 16.11-16.12).

$(NH_4)_2PtCl_6$ and $PtCl_2$. These inorganic Pt-compounds did not induce apoptosis both at early stages of this process as detected by the annexin V/PI assay (Figures 16.9-16.10 and 16.13-16.14, respectively) and at later stages as confirmed by the DNA fragmentation and the Hoechst 33342/PI methods (Photos 16.15-16.18, 16.23-16.26 and 16.32, 16.34, respectively). However, the case of $(NH_4)_2PtCl_6$ at 100 μM (6-hour exposure) and 75 μM (12-hour exposure) as well as the case of $PtCl_2$ at 75 μM (6- and 12-hour exposure) demonstrated induction of caspase-3 activity with values ranging from about 5% to 15% for both metal compounds (Tables 16.X and 16.XII, respectively).