3. RESULTS

3.1. Bisphosphonates inhibit melanoma cell proliferation *in vitro*

Bisphosphonates have been shown to inhibit the cell proliferation in different types of human tumour cells. In order to investigate the effect of bisphosphonates on melanoma cell growth, the melanoma cell lines A375 and M186 were treated with increasing concentrations of pamidronate, zoledronate and clodronate for 24 h. The number of cells was then measured using the crystal violet method as described previously (WIEDER *et al*., 1998). Briefly after treatment, cells were fixed and incubated with crystal violet, a dye which bounds to DNA of the cells. After washing off the dead cells, the bound dye was extracted from the adherent cells with Triton-X and then quantitatively analysed with an ELISA photometer. As shown in figure 4, in both cell lines, pamidronate as well as zoledronate treatment resulted in a dose-dependent decrease in cell number. In A375 (Fig. 4a) a significant reduction in cell number was observed after treatment with 50 µM pamidronate, and reached the maximum after treatment with 100 µM pamidronate (84 %). Higher concentrations of pamidronate were not able to induce further decrease in cell number. Zoledronate was able to significantly inhibit cell growth starting at the concentration of 30 µM and reaching a maximal decrease in cell number (45% of control) at the concentration of 100 µM.

A similar effect of bisphosphonates treatment was observed in M186 cells (Fig. 4b). A significant reduction of cell number was observed for pamidronate at a concentration of 100 µM (93 %) and for zoledronate beginning at the concentration of 30 µM, with a maximal effect at 100 µM (57.35 % of control).

In contrast, incubation of both A375 and M186 cells with the non-amino-bisphosphonate clodronate, at concentrations ranging from 100 µM to 1000 µM, failed to induce a significant decrease in cell number within 24 h, even at the highest doses.

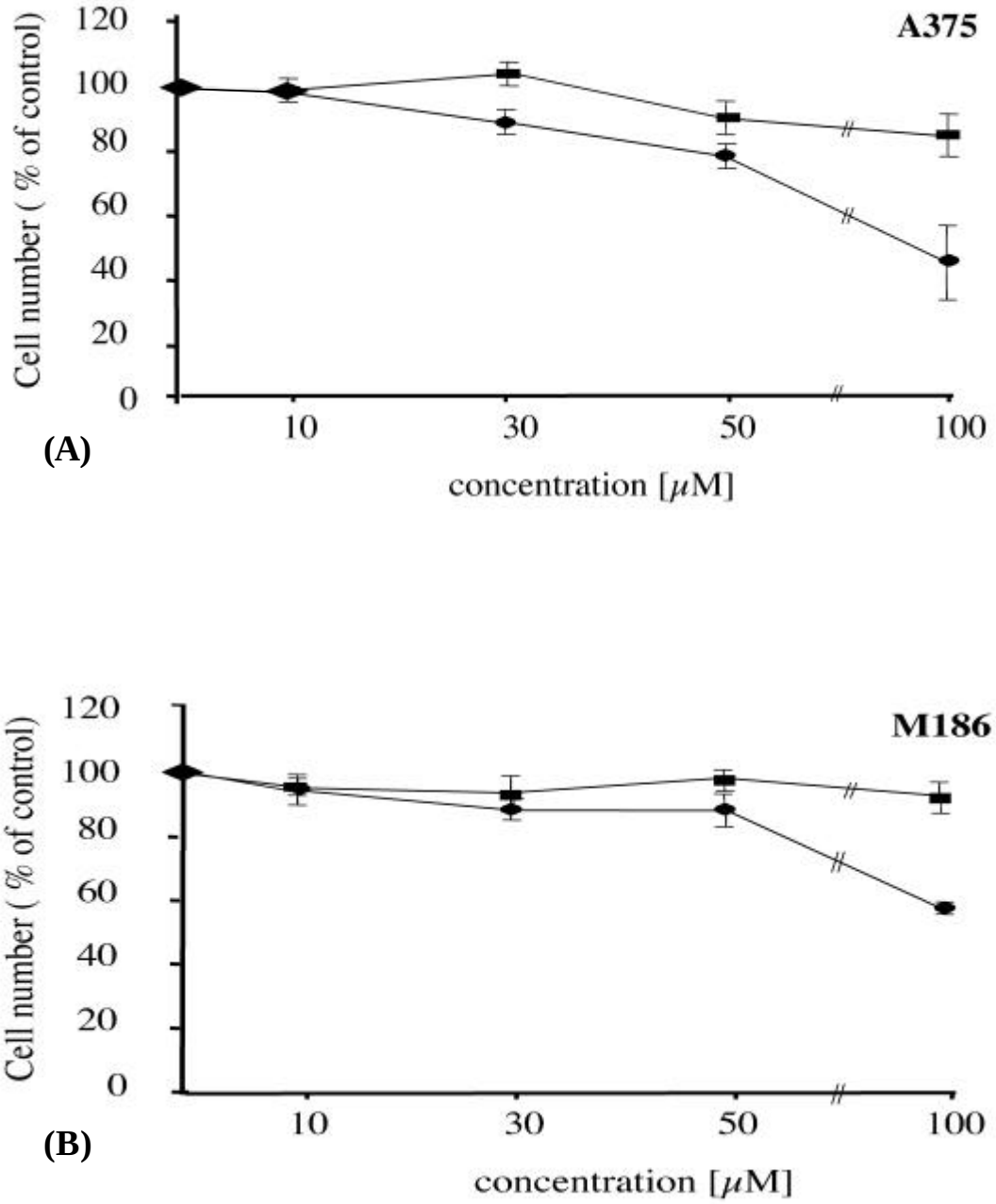

Figure 4. Effect of bisphosphonates on proliferation of melanoma cells *in vitro*.
A375 (a) and M186 (b) melanoma cells were seeded at a density of 30,000 cells /cm^2, allowed to adhere over night and then treated with the indicated concentrations of pamidronate (squares), zoledronate (diamonds) or vehicle for 24 h. Proliferation was then measured using the crystal violet staining of adherent cells. Growth of control cells was set at 100 % and the cell number of treated cells was calculated as percentage of control. Values represent the mean value of four experiments ± SD

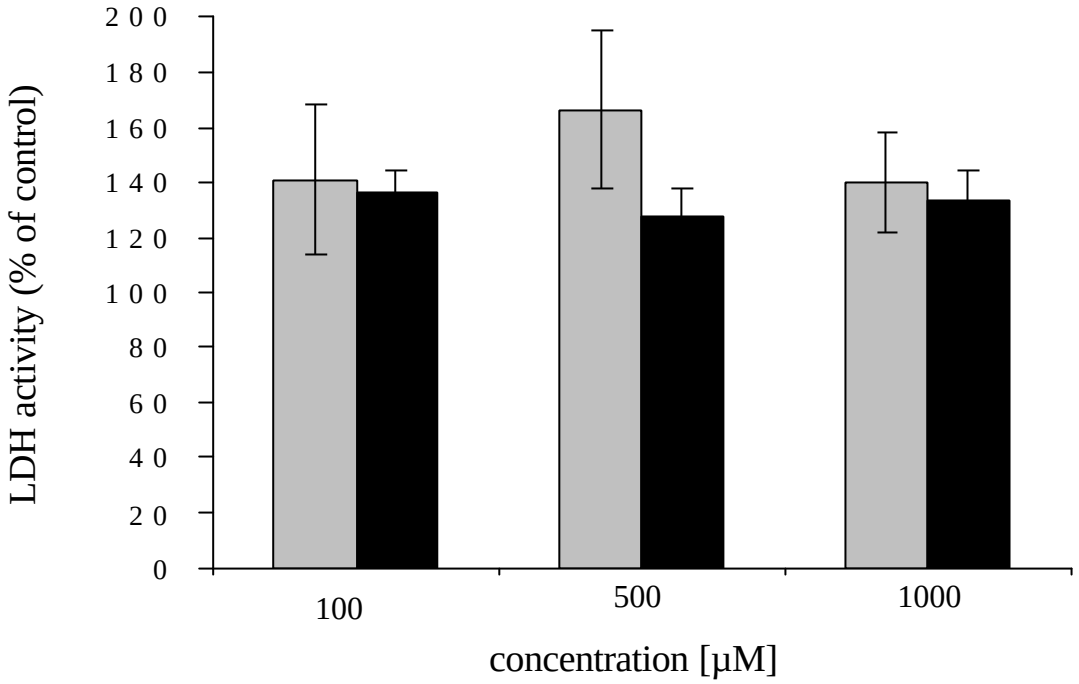

Figure 5. Cytotoxic effect of clodronate in melanoma cells *in vitro*.
A375 (light grey columns) and M186 (black columns) melanoma cells were seeded at a density of 40,000 cells/cm^2 and left over night to adhere. The next day they were treated with the given concentrations of clodronate or vehicle for 24h. Cytotoxicity was measured as increase of LDH activity in the cell culture supernatant. LDH activity in the supernatant of control cells was set at 100 % and the LDH release in treated cells was calculated as percentage of control. Values represent the mean of four experiments ± SD.

3.2. Bisphosphonates have no cytotoxic effect in melanoma cells *in vitro*

A possible unspecific cytotoxic effect of bisphosphonates on melanoma cells was investigated by measuring the extracellular release of lactate dehydrogenase (LDH), following bisphosphonate treatment. LDH is a ubiquitous cytoplasmatic enzyme, which is released from cells after plasma membrane damage. An increase of LDH activity measured in cell culture supernatant signifies increased release of the enzyme and is thus a marker for cell necrosis.

M186 and A365 cells were incubated for 24h with pamidronate, zoledronate, and clodronate. No increase of LDH release in comparison with controls was found in M186 and A375 cells treated for 24 hours with zoledronate in concentrations range between 10 and 100 µM. The treatment of A375 and M186 cells with pamidronate 100 µM for 24 h resulted in a slight, non-significant increase in LDH release, most probably as a result of secondary necrosis.

In contrast, after treatment with the non-amino-bisphosphonate clodronate, in concentrations ranging from 100 µM to 1000 µM, a moderate but significant increase in the LDH activity could be measured at 24h for all the concentrations used (Fig.5).

Clodronate seemed thus to be able only to generate a low grade of cell necrosis, but to have no apoptotic effect in melanoma cells and consequently no significant influence upon the cell growth at the studied doses.

3.3. Bisphosphonates induce apoptosis in human melanoma cells *in vitro*

A key feature of apoptosis is the controlled cleavage of genomic DNA by specific endogenous endonucleases at internucleososmal sites, resulting in histone-bound DNA fragments, which are released in the cytoplasma of cells. The amount of mono-/ oligo nucleosomes thus released into the cytoplasma can then be measured by means of an ELISA assay, in order to achieve a quantification of apoptosis.

DNA fragmentation as mark of apoptosis was evaluated in A375 and M186 cells after 24 h of incubation with increasing concentrations of pamidronate, zoledronate and clodronate respectively.

A dose-dependent induction of DNA fragmentation was seen after both zoledronate and pamidronate treatment in the two cell lines studied. In A375 cells, a significant effect was detectable at concentrations of 50 µM and reached a maximum at 100 µM (Fig 6 a, b). In M186 cells, DNA fragmentation was detected at a concentration of 30 µM, and showed a concentration-dependent increase, reaching the maximum at 100 µM.

In both cell lines, pamidronate had a stronger effect in inducing apoptosis, with DNA fragmentation reaching 711 % of controls in A375 cells treated with 100 µM pamidronate, and 746 % of control in M186 cells at the same concentration. DNA fragmentation for 100 µM zoledronate in contrast, was only 280 % of controls in A375 cells and 247 % in M186 cells respectively.

In order to assess the effect of the non-amino-bisphosphonate clodronate on induction of apoptosis, A375 and M186 cells were treated for 24 h with clodronate in concentrations ranging from 100 µM to 1000 µM. Only a slight increase in DNA fragmentation (137 % of controls) was observed in M186 cells treated with the highest dose of clodronate (1000 µM). Moreover, in A375 cells, clodronate in the same concentrations range showed no effect on induction of DNA fragmentation (Fig 6 c).

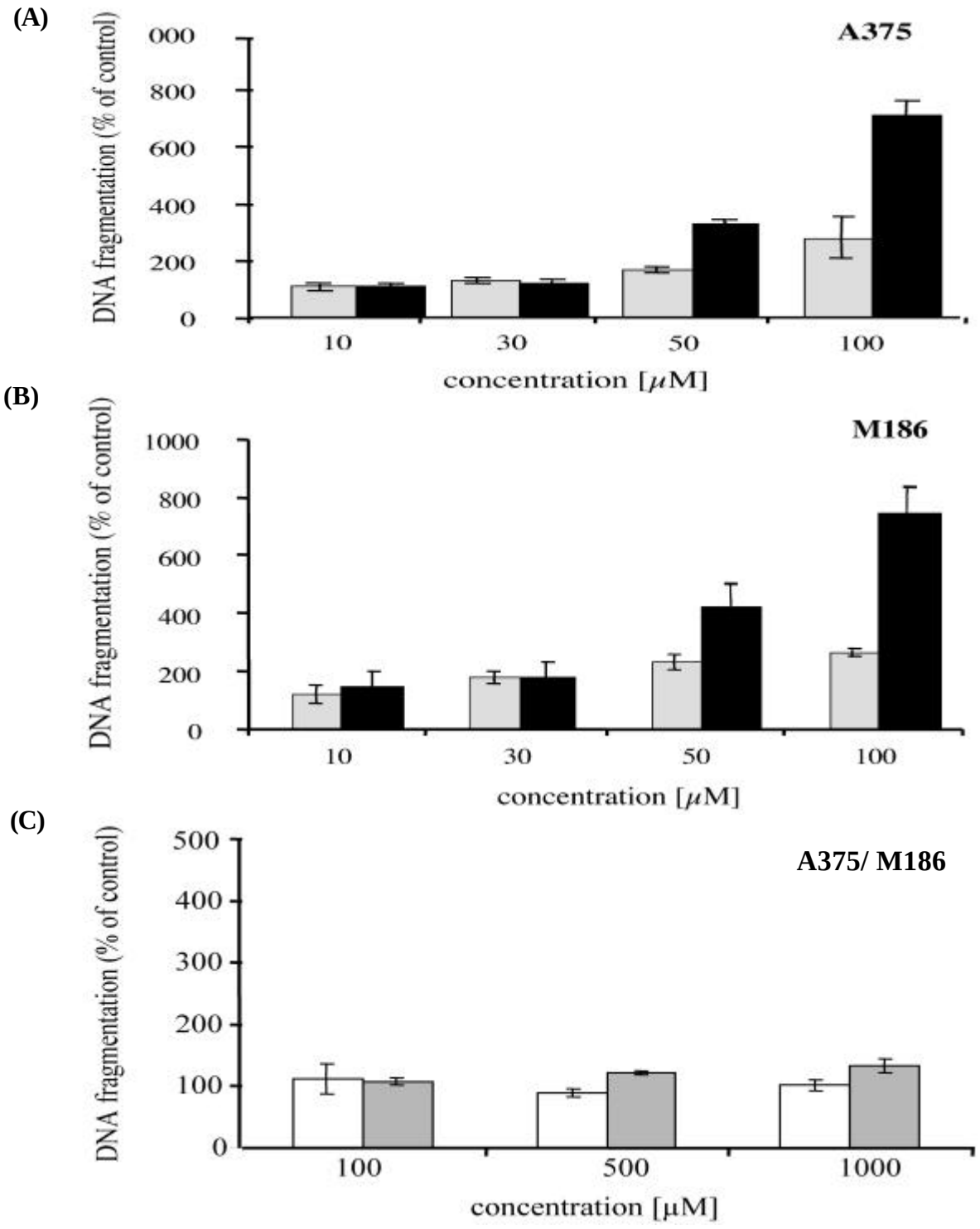

Figure 6. Effect of bisphosphonates on the induction of apoptosis in melanoma cells. A375 (A) and M186 (B) melanoma cells were seeded at a density of 40,000 cells/cm^2 and left over night to adhere. The next day they were treated with the indicated concentrations of zoledronate (grey columns), pamidronate (black columns) or vehicle for 24 h. DNA fragmentation was measured using an ELISA assay detecting cytoplasmic nucleosomes.
(c) A375 (grey columns) and M186 (black columns) cells were treated for 24h with clodronate in the indicated concentrations. Apoptosis was evaluated by measuring the DNA fragmentation. DNA fragmentation of control cells was set at 100 % and the DNA fragmentation in treated cells was calculated as percentage of control. Values represent the mean of four experiments ± SD

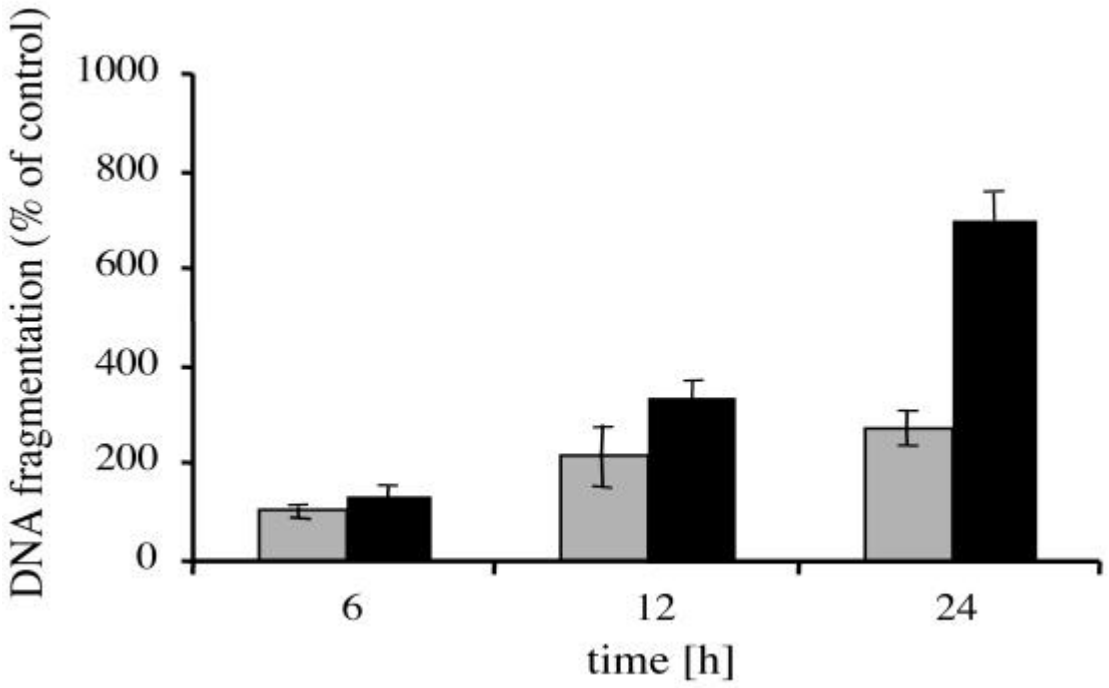

Figure 7. Time dependency of bisphosphonates-induced apoptosis in melanoma cells. A375 melanoma cells were seeded at a density of 40,000 cells/cm^2 and left over night to adhere. The next day they were treated with the indicated concentrations of zoledronate (grey columns), pamidronate (black columns) or vehicle for 6,12 and 24 h respectively. At each time point, DNA fragmentation was measured using an ELISA assay detecting cytoplasmic nucleosomes. DNA fragmentation of control cells was set at 100 % and the DNA fragmentation in treated cells was calculated as percentage of control. Values represent the mean of four experiments ± SD

3.3.1. Bisphosphonates induce apoptosis in melanoma cells *in vitro* in time-dependent manner

In order to study the relationship between the apoptotic effect of bisphosphonates and the duration of treatment, A375 melanoma cells were incubated for 6, 12, 24 hours with 100 µM pamidronate and zoledronate respectively. At each time point DNA fragmentation was measured (Fig.7). An increase in DNA fragmentation could first be seen after 12 hours of incubation with the bisphosphonates, and increased further markedly after 24 hours of treatment, up to 700 % of controls for pamidronate and 315 % for zoledronate. Thus, the apoptosis induced by bisphosphonates is dependent on both concentration and duration of treatment.

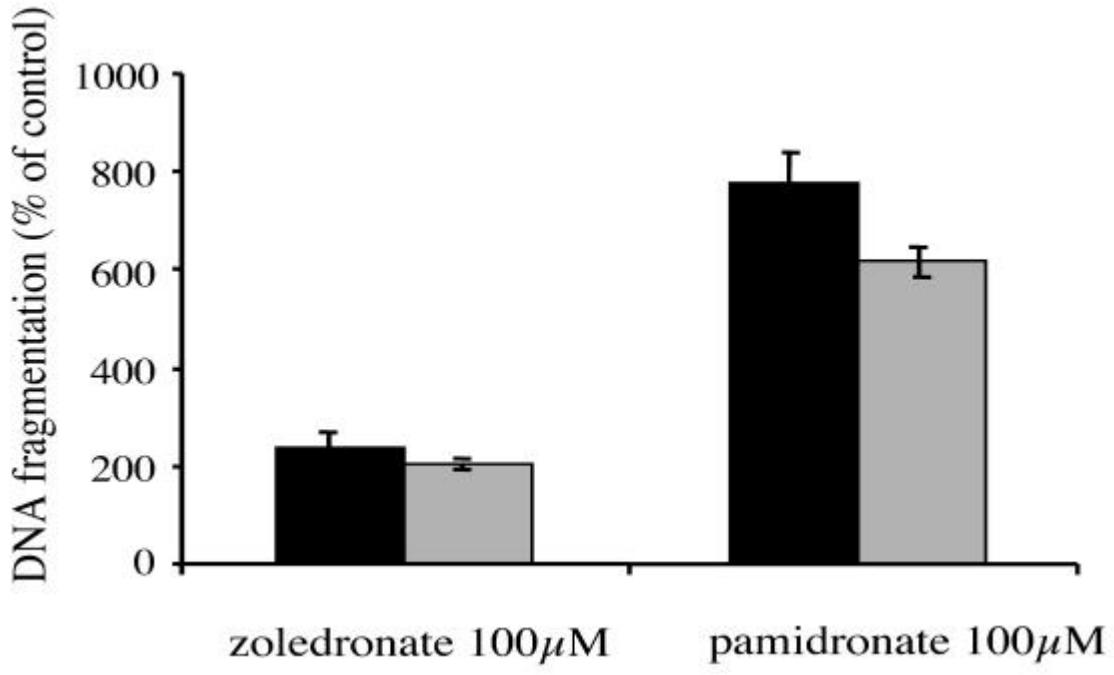

Figure 8. Induction of apoptosis by bisphosphonates in Bcl2-overexpressing melanoma cells. A375/Bcl-2 (dark columns) and A375/pIRES (light columns) cells were seeded at a density of 40.000 cells/cm2 and left over night to adhere. The following day they were treated with the indicated concentrations of zoledronate, pamidronate or vehicle for 24 h. DNA fragmentation was measured using an ELISA assay detecting cytoplasmatic nucleosomes. DNA fragmentation of control cells was set at 100 % and the DNA fragmentation in treated cells was calculated as percentage of control. Values represent the mean of four experiments ± SD.

3.3.2 Bcl-2 overexpression does not prevent bisphosphonate-induced apoptosis in cultured melanoma cells

The Bcl-2 protein has been demonstrated to confer the cells resistance against different pro-apoptotic stimuli. Moreover, shifting the ratio of antiapoptotic protein Bcl-2 to the proapoptotic protein Bax is one of the mechanism by which tumor cells can escape death induced by stimuli acting on the mitochondrial pathway of apoptosis (BOISE *et al*, 1993). The importance of a high Bcl-2/ Bax ratio for the antiapoptotic protection and enhancement of cell survival has also been demonstrated in melanoma cells (RAISOVA *et al*, 2001).

In order to examine the influence of Bcl-2 upon bisphosphonate-induced apoptosis in melanoma cells, A375/Bcl-2 cells which stably overexpress Bcl-2 , and A375 cells transfected with the empty vector only (A375/pIRES) as control, were treated for 24 h with 100 µM pamidronate and zoledronate respectively.

As shown in figure 8, overexpression of Bcl-2 could not protect the cells from the apoptosis induced by the two bisphosphonates. Both Bcl-2-overexpressing melanoma cells and the cells transfected with empty vector showed similar sensitivity to bisphosphonates. Treatment with pamidronate induced an increase in DNA fragmentation of 617 % of control in A375/pIRES and of 776 % of controls in A375/Bcl-2. As shown previously in A375 cells, zoledronate was less effective in inducing apoptosis, achieving an increase in DNA fragmentation to 150 % compared to controls in A375/pIRES, and to 200 % of control in A375/Bcl-2.

3.3.3. Effect of short-time exposure of melanoma cells to bisphosphonates

As previously shown, 12 h and 24 h treatment with bisphosphonates results in apoptosis induction in melanoma cells *in vitro*. However, in clinical conditions, due to the rapid clearance from the circulation and bone entrapment of bisphosphonates, tumor cells with extraosseous localisation would be probably exposed to these drugs only for few hours after administration. Therefore, in order to examine a model, which may more accurately reflect the situation *in vivo,* the effect of 6 hours exposure to 100 µM pamidronate on induction of apoptosis in A375, A375/pIRES and A375/Bcl-2 cells was investigated.

The three melanoma cell lines were incubated for 6 hours with 100 µM pamidronate or vehicle as control. After 6 hours, the drug-containing medium was removed, the cells were rinsed in PBS and then supplemented with fresh culture medium and further incubated for another 18 hours. Then DNA-fragmentation was measured and compared to that obtained in the same cell lines continuously incubated over 24 h with 100 µM pamidronate. The results are shown in figure 9.

It was interesting to note that in A375 and A375/pIRES cells, exposure to a 6 h pulse of pamidronate resulted in almost the same effect on DNA fragmentation (measured after 24 h) as the continuous incubation for 24h with the compound. In contrast, as shown previously, (Fig 7) in A375 cells no significant increase in DNA fragmentation was observed when measured directly after 6 hours incubation with 100 µM pamidronate.

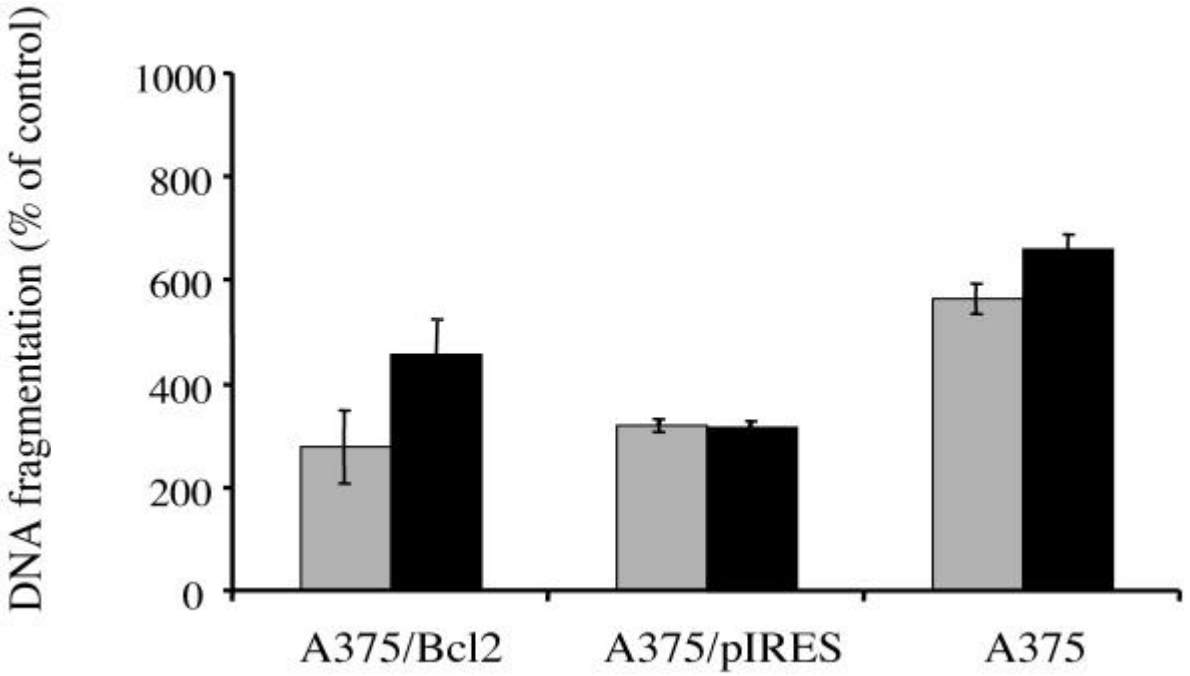

Figure 9. Effect of short term exposure of melanoma cells to pamidronate
A 375, A375/Bcl-2 and A375/pIRES cells were seeded at a density of 40,000 cells /cm^2 and left over night to adhere. On the following day they were either incubated with 100 µM pamidronate for 6 h, after which the drug containing medium was replaced with fresh culture medium and the cells were further incubated for 18 h (grey columns), or continuously incubated with the drug for 24 h (black columns). DNA fragmentation was measured using an ELISA assay detecting cytoplasmatic nucleosomes. DNA fragmentation of control cells was set at 100 % and the DNA fragmentation in treated cells was calculated as percentage of control. Values represent the mean of four experiments ± SD.

In Bcl2-overexpressing melanoma cell line A375/Bcl-2, DNA fragmentation reached 280 % of control after the short time exposure to pamidronate and 450 % after continuous incubation with the drug over 24 hours. Thus, although Bcl-2 overexpression could not protect cells from apoptosis induced by 24 h incubation with pamidronate, it seemed however to have a certain protective effect, in the conditions of a shorter-term exposure to the drug.

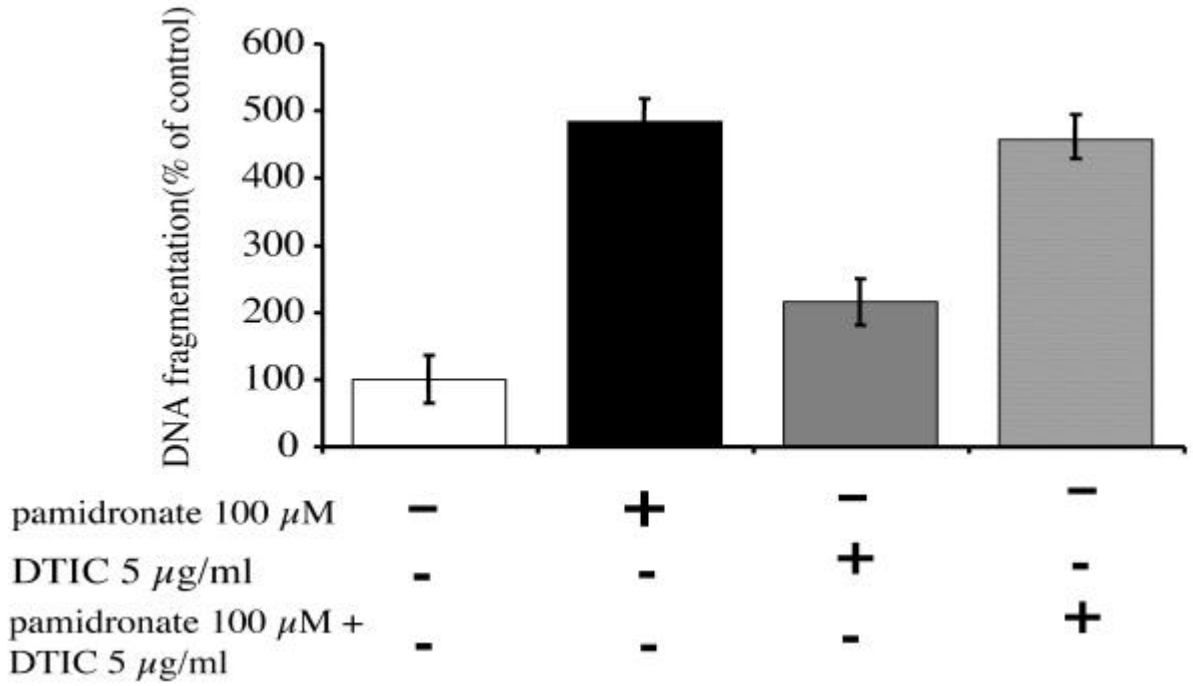

Figure 10. The apoptotic effect of combination of pamidronate with DTIC in cultured melanoma cells. A375 melanoma cells were seeded at a density of 40,000 cells/cm^2 and left over night to adhere. The following day they were treated with 100 µM pamidronate (black column), 5 µg /ml DTIC (dark grey column) or both (light grey column) for 24 h. DNA fragmentation of control cells (white column) was set at 100 % and the DNA fragmentation in treated cells was calculated as percentage of control. Values represent the mean of four experiments ±SD

3.3.4. Effect of combination of pamidronate and DTIC in melanoma cells

In present different drugs are used in the chemotherapy of malignant melanoma and have been proven to induce apoptosis in melanoma cells. In order to evaluate better the potential role of bisphosphonates in the adjuvant antitumor therapy, it would be interesting to study the effect of combinations between bisphosphonates and these classical chemotherapeutics.

DTIC (dacarbazine) is an alkylating agent, which acts through induction of apoptosis in tumor cells and is currently used as standard chemotherapy in melanoma. In order to investigate a possible synergistic or additive effect of pamidronate and DTIC, A375 and M186 cells were incubated for 24 h with 100 µM pamidronate, 5µg /ml DTIC, or with pamidronate plus DTIC respectively. The results were similar in the two cell lines studied; the data of A375 are shown in figure 10.

The results of this experiment indicate that, at the concentrations used, pamidronate has *in vitro* a stronger proapoptotic effect as DTIC, inducing a degree of DNA fragmentation of 460 % of control, while the apoptosis induction in the cells treated with DTIC was only 230 % of control. In contrast, the combination of pamidronate plus DTIC, at the given concentrations, showed no synergistic nor additive effect in apoptosis induction in the melanoma cells studied. Treatment with pamidronate plus DTIC resulted in almost the same effect on inducing DNA fragmentation as pamidronate alone.

3.3.5. Cytochrome c release is not involved in bisphosphonate- induced apoptosis *in vitro*

It has been demonstrated that mitochondria plays a pivotal role in apoptosis induced by many stimuli, by coordinating the caspase activation through release of cytochrome c. Cytochrome c and many other proteins, normally confined to the intermembrane space of the mitochondria are released during apoptosis. The release of mitochondrial proteins in turn has been showed to be controlled by the Bcl-2 family members, whereby overexpression of antiapoptotic proteins such as Bcl-2 or Bcl-X_L in many cell types exposed to various apoptotic stimuli, prevents cytochrome c release, caspase activation and cell death.

In order to investigate a possible involvement of the mitochondrial pathway in the bisphosphonate-induced apoptosis in melanoma, cytochrome c release in Bcl-2 overexpressing melanoma cells A 375/ Bcl-2 and in the empty vector transfected cells (A 375/ pIRES), after treatment with pamidronate was analysed.

Cells were incubated for 16 h with 100 µM pamidronate, after which the cells were harvested, the cytosolic fraction was isolated as described (chapter 2.2.4) and the presence of cytochrome c in the cytoplasmatic compartment was determined by Western blot analysis. As positive control, the cytosolic extracts of HL-60 cells, treated for with for 5 h was included.

As shown in figure 11, the treatment with pamidronate did not result in an increase of the cytochrome c in the cytoplasma. Similarly, no cytochrome c release could be demonstrated in cells incubated for 24h with pamidronate (data not shown).

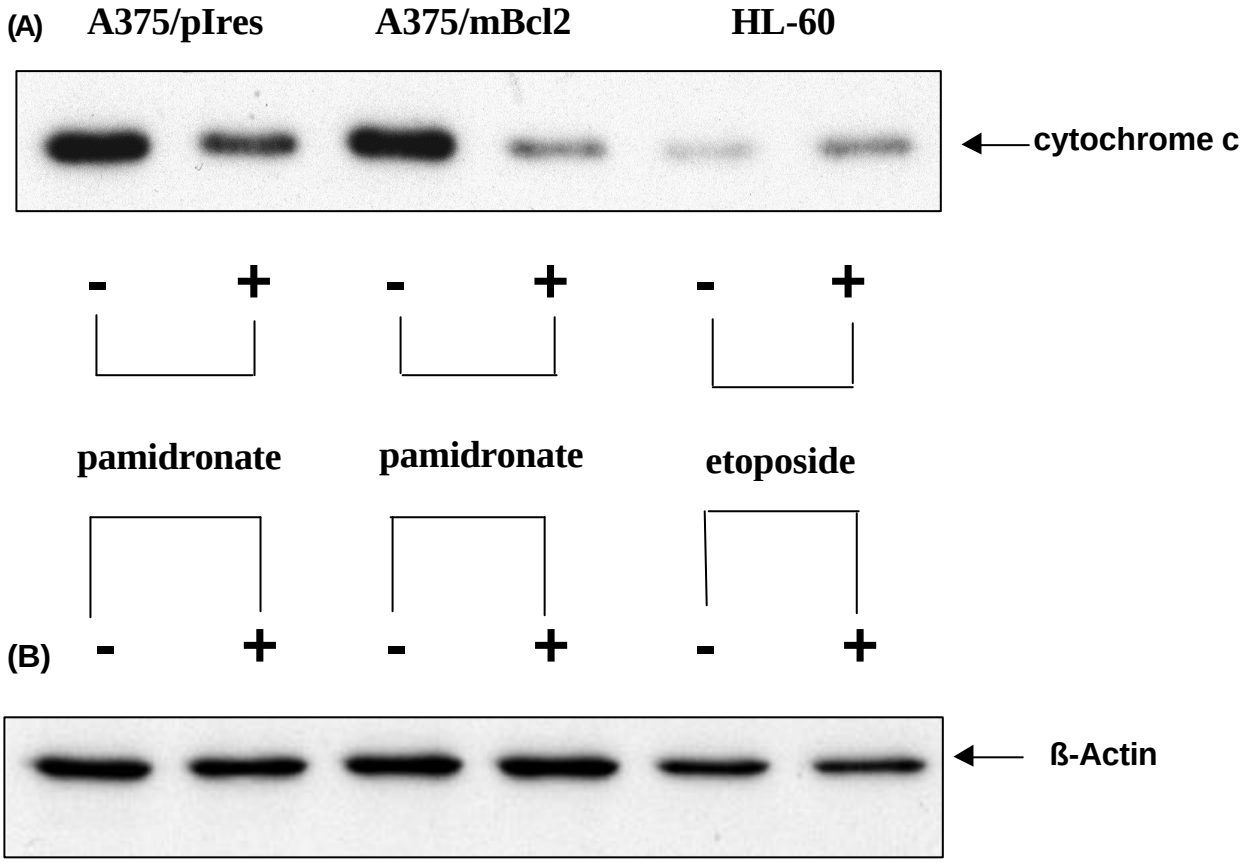

Figure 11. Effect of pamidronate on cytochrome c release in melanoma cells
A375/ bcl2 and A375/ pIRES cells were seeded at 20 000/cm^2 and after 48h treated with 100 µM pamidronate or vehicle. After 16 h incubation with the drug, cells were harvested, cytosolic and mytochondrial fractions were isolated as described (chap 2.2.4) and cytochrome c in each fraction was analyzed by Western blot. HL-60 cells treated with etoposid for 5 h were used as positive control. Anti-human cytochrome c antibodies were used. Cytosolic fraction: (A) cytochrome c; (B) actin

3.4. Effects of bisphosphonates on cell cycle progression in melanoma cells *in vitro*

Tumor cells are proliferating cells which cycle between the four phases of the cell cycle: G1, S, G2 and M, during which the DNA content increases progressively until it is doubled. By measuring the DNA content of a cell is therefore possible to determine in which phase of the cycle the cell is.

Different studies have shown that bisphosphonates are able to induce cell cycle alterations in different types of human tumor cells *in vitro*, which could explain at least in part the inhibitory effect of these compounds upon tumor cell growth. For melanoma cells no data were available up to now.

In order to study the effect of bisphosphonates on cell cycle of melanoma cells, FACS analysis of DNA content was use to study the distribution in cell cycle phases of A375 and M186 cells lines. The cells were treated over 24 hours with increasing concentrations of pamidronate, zoledronate, and clodronate respectively.

The control cultures showed a distribution of cells in the cell cycle phases typical for proliferating cells, with an average of 61 % of cells having a 2n DNA content, corresponding to G0/G1 phase, 10 % a 4n DNA content (G2/M), and 28 % showing a DNA content between 2n and 4n, corresponding to the S phase.

Cells treated with pamidronate and zoledronate in a concentration range of 10 µM to 100 µM showed comparable dose-dependent alterations in this distribution, with an increase of number of cells in S phase accompanied by a reduction in the proportion of cells in G0-G1 and G2-M phases (fig 12a-b). Zoledronate was more potent in inducing cell cycle changes, its effects starting at concentrations of 30 µM, while pamidronate significantly altered the distribution of cells in the cell cycle phases only at the highest concentration. The maximal effect was seen for both drugs at the concentration of 100 µM, with an increase in the proportion of cells in S phase from 28 % to 46 % for pamidronate and to 47.5 % for zoledronate (Table 8).

In contrast, treatment of cells with clodronate at 10 x higher concentrations showed a different pattern, with a slight increase in the proportion of cells in G0/G1 phase at the maximal dose (1000 µM), which was accompanied by a corresponding moderate decrease in cells in S- and G2/M- phase (Fig 12 c).

As control, the effect of treatment with 5 mM hydroxyurea over 24 h on the cell cycle progression in A375 and M186 cells is also shown (Table 8). Hydroxyurea is a standard chemotherapeutic agent in melanoma, with an established effect in inducing G1 arrest in melanoma cells. Accordingly, the DNA content analysis clearly showed an increase of the proportion of cells in G1, with a corresponding decrease in cells in S and G2/M phase in the melanoma cells treated with this drug.

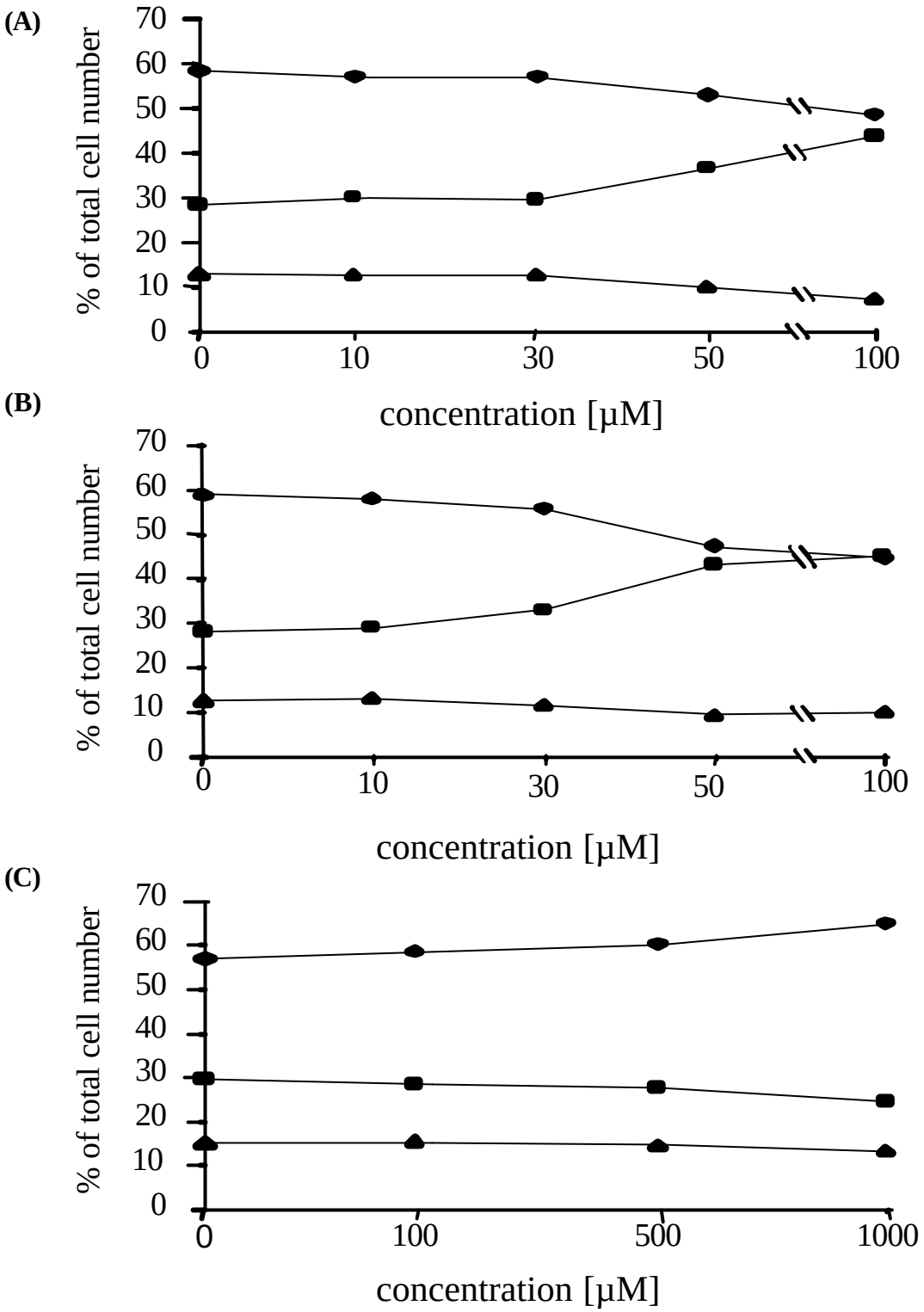

Figure 12. Distribution in various phases of the cell cycle of cultured melanoma cells after bisphosphonate treatment. A 375 and M186 cells were seeded at 80,000 cells/well in 6 wells plate and left to adhere. After 36 h they were treated with pamidronate, zoledronate, clodronate or vehicles in the indicated concentrations for 24 h. The percentage of A375 cells in G0/G1 (diamonds), S (squares) and G2/M (triangles) phase of the cell cycle, after treatment with (A) pamidronate, (B) zoledronate, (C) clodronate, is shown. The DNA histograms were created using ModFit LT software (version 2.0).

Table 8. Effects of bisphosphonates on the cell cycle progression in melanoma cells. A375 and M186 cells were incubated for 24 h with pamidroante, zoledronate, clodronate, and hydroxyurea in the indicated concentrations. The cells were then harvested, fixed in 70% ethanol and stained with propidium iodide. The DNA content was then analysed by FACS. Data represent the percentage of cells in G0/G1, S and G2/M phases as determined by ModFitLT software. Values are mean of 2 independent experiments

	TREATMENT	G1 (%)	S (%)	G2/M (%)
A375				
	control pamidronate	61.29	28.25	10.465
	pamidronate 10 µM	60.06	29.805	10.135
	pamidronate 30 µM	60.63	29.35	10.02
	pamidronate 50 µM	57.025	32.615	10.365
	pamidronate 100 µM	46.905	46	7.1
	control zoledronate	63.81	26	9.685
	zoledronate 10 µM	60.92	29.68	9.4
	zoledronate 30 µM	55.985	38.815	5.2
	zoledronate 50 µM	47.59	46.81	5.6
	zoledronate 100 µM	44.025	47.475	8.5
	control clodronate	62.15	26.69	10.15
	clodronate 100 µM	62.715	27.125	10.16
	clodronate 500 µM	63.955	25.515	10.535
	clodronate 1000 µM	65.36	23.35	11.28
	hydroxyureea 5mM	76.7	16.8	6.8

	TREATMENT	G1 (%)	S (%)	G2/M (%)
M186				
	control pamidronate	58.38	28.48	13.14
	pamidronate 10 µM	57.25	30.025	12.715
	pamidronate 30 µM	57.14	30.04	12.82
	pamidronate 50 µM	53.13	36.725	10.155
	pamidronate 100 µM	48.58	43.905	7.51
	control zoledronate	59.005	28.15	12.84
	zoledronate 10 µM	57.86	29.16	12.98
	zoledronate 30 µM	55.81	33.015	11.675
	zoledronate 50 µM	47.42	43.22	9.36
	zoledronate 100 µM	44.51	45.305	10.185
	control clodronate	54.595	28.645	15.255
	clodronate 100 µM	56.095	28.51	15.4
	clodronate 500 µM	57.745	27.605	14.655
	clodronate 1000 µM	62.395	24.4	13.205
	hydroxyureea 5mM	73.37	20.46	6.17