4. DISCUSSION

Melanoma is at present one the most malignant human tumors. Its increasing incidence, its high prevalence in young age, its aggressive biological behaviour and its high mortality rates in advanced stages make it a major medical problem. Despite intensive efforts in clinical research, the existing therapeutic options for metastatic melanoma are disappointing, being associated with a very poor response rate and high systemic toxicity. Therefore the search for new substances active against cancer and for new treatment strategies is required.

One of these new alternatives for the anticancer therapy could be represented by the bisphosphonates. Bisphosphonates are a class of powerful inhibitors of osteoclast-mediated bone resorption, which are currently associated with standard chemo- and hormonal therapy for the treatment of metastatic bone disease.In contrast to other classical antineoplasic agents, they are very well tolerated even in prolonged administration. Recently it has been demonstrated that these compounds may also have a direct antitumor effect, preventing tumor cell growth and invasion in different experimental *in vitro* and *in vivo* models, so that bisphosphonates became the subject for intensive research as potential candidates for developing new treatment approaches for human tumors. In melanoma however, no data about a the effect of bisphosphonates are available, so that the aim of the present study was to explore the influence of these compounds on cell proliferation and cell survival in melanoma cell lines *in vitro,* and to obtain new indices concerning their intracellular mechanism of action.

Choice of drugs

A broad range of bisphosphonates are now available for the clinical use, and new compounds have been continuously developed in order to achieve a higher antiresorptive potency; the newest members of this drug family, such as zoledronate, are considered to be about 10,000 times more effective in inhibiting bone demineralisation than the first generation of bisphosphonates. A close relationship between the molecular structure of the bisphosphonates and their antiresorptive potency has been observed, and it is now accepted that the structure determines the antiresorptive potency by influencing both the affinity to bone and pharmacological properties of the drugs, as well as their intracellular mechanism of action. Accordingly, two main pharmacological classes of bisphosphonates are described: the amino-bisphosphonates, with high antiresorptive potency, who seem to act by inhibiting the enzymes of

mevalonate pathway and by inactivating signalling proteins such as small GTPases; and non-amino-bisphosphonates, less potent, who can be metabolised to toxic analogues of ATP.

However, the relationship between antiresorptive potency, mechanism of action and cellular effects of bisphosphonates is not yet completely elucidated. It is not yet clear to which extent compounds of different pharmacological class also differ in their effects in tumor cells, nor if higher antiresorptive potency implies also a stronger effect against tumor cell growth.

In order to answer these questions, three bisphosphonates were chosen for this study: clodronate, a non-amino bisphosphonate and one of the first developed derivates, and two amino-bisphosphonates: pamidronate, which is widely used in clinical practice, and the newly developed zoledronate. Pamidronate is considered to be about 100 times more efficient as clodronate in inhibiting bone resorption in vivo. Zoledronate is the most potent anti-resorptive agent known to date, being reported by different studies to be 100 to 1000 times more active against bone demineralisation than pamidronate, both in animal models and in clinical trials.

Effects of bisphosphonates on cell proliferation and induction of apoptosis in melanoma cells in vitro.

The present study demonstrated that both amino-bisphosphonates are able to decrease cell proliferation *in vitro* in a dose dependent manner. This effect could be partially due to induction of apoptosis, as both pamidronate and zoledronate induced DNA fragmentation in the melanoma cells studied. Moreover, inhibition of cell growth was not the result of cell necrosis, as no significant release of LDH from the cells was measured after treatment with the two bisphosphonates.

In contrast, the non-amino-bisphosphonate clodronate, even at concentrations 10 times higher, had no significant effect on the cell number and induction of apoptosis in cultured melanoma cells. In higher dose, clodronate caused only a slight increase in LDH activity, suggesting some cytolytic effect.

These differences observed in the activity of the three bisphosphonates may reflect the difference in the mechanism of action between the amino- and non-amino bisphosphonates. The stronger anti-proliferative and/or pro-apoptotic effect of amino-bisphosphonates compared to non-amino-bisphosphonates was also reported in other cell types such macrophages (ROGERS *et al*.,1999; BENFORD *et al*.,1999), breast cancer cells (SENERATNE *et al*., 2000), multiple myeloma (SHIPMAN *et al*.,2000) or colonadenocarcinoma (SURI *et al*., 2001), and this effect appears to be related to the ability of amino-bisphosphonates to inhibit protein prenylation.

Prenylation is a functional requirement for signalling proteins such as small GTPases. Among these, the Ras and Rho subfamilies of GTP-ases are well studied and have been shown to mediate key cellular processes in response to various external stimuli, such as cell growth, apoptosis, lipid metabolism, cytoarchitecture, and transcriptional regulation (AZNAR et LACAL, 2001). In the same time, their constitutive activation (by point mutations or overexpression) escapes the regulatory signals, and leads to uncontrolled cell growth, enhanced angiogenesis, inhibition of apoptosis and genetic instability, which are essential features of tumor development. (BAR-SAGI, 2000). The important contribution of aberrant Ras activation in oncogenesis is well established. Similarly, overexpression of Rho proteins have been demonstrated in different human tumors, including melanoma (FRITZ *et al*., 1999; CLARK *et al*., 2000). Moreover, in melanoma, ectopic overexpression of RhoC in A375 cell line was sufficient to create a highly metastatic phenotype (CLARK *et al*., 2000). Therefore, inactivation of small GTPases, via inhibition of prenylation, could explain at least in part the inhibitory effects of nitrogen-containing bisphosphonates on tumor cells in general and in melanoma in particular.

In contrast to amino-bisphosphonates, clodronate does not inhibit the isoprenoid synthesis (RESZKA *et al*., 1999). In change, it has been reported that non-amino-bisphosphonates can induce cell death by metabolization to toxic non-hydrolysable analogues of ATP (ROGERS *et al*., 1996). The inactive ATP analogues may disrupt the energy-requiring processes of the cells and induce cell death. In the present study however, clodronate even at high concentrations had no effect on the cell survival *in vitro*, suggesting that this mechanism has no functional significance in melanoma cell lines.

Both inhibition of proliferation and induction of apoptosis in cultured melanoma cells by the two amino-bisphosphonates were observed at concentrations ranging from 10 μM to 100 μM. Similar concentrations of amino-bisphosphonates have been reported to act antiproliferative and/or proapoptotic in other types of tumor cells, e.g. myeloma (SHIPMAN *et al*.,1997), breast cancer (SENERATNE *et al.,* 2000) or prostate cancer (LEE *et al*., 2001). It is however not clear if these high doses may also be achieved *in vivo*, at least using the current dosage and treatment regimens. It is known that following administration, bisphosphonates are rapidly removed from circulation and accumulate in the bone, so that primary tumor cells or visceral metastases would be probably exposed to only much lower doses of bisphosphonates. Indeed, the peak serum level for amino bisphosphonates has been reported to range between 1 and 5 μM, depending on the route of administration. (BERENSON *et al*., 1997; DALEY-YATES *et al*., 1991). In contrast, due to their affinity for hydroxyapatite and to their accumulation at the

active sites of bone resorption, bisphosphonates may reach concentrations as high as 1000 μM within the bone. (SATO *et al* 1991; APARICIO *et al*.,1998). This local accumulation in bone could explain the selective effect of bisphosphonates on bone metastases reported by different *in vivo* studies (HIRAGA *et al*., 2001). Therefore, in order to achieve effective concentrations also in tissues, new compounds, with less affinity for bone will have to be designed.

The effects of the amino-bisphosphonates on proliferation and induction of apoptosis and in melanoma cells showed a concentration dependency. For both compounds, a significant increase of DNA fragmentation was observed in the studied melanoma cell lines starting at concentration of 50 μM and the effect increased to a maximum at 100 μM. An inhibition of proliferation was demonstrated at the concentration of 30 μM (for zoledronate), and 50 μM for pamidronate, and the effect also increased with the concentration, up to a maximum at 100 μM.

Pamidronate was more efficient than zoledronate in inducing apoptosis in both cell lines studied, causing an increase in DNA fragmentation up to about 700% of controls at 100 μM. At the same concentration, DNA fragmentation induced by zoledronate reached only 300% of controls.

In contrast, zoledronate proved to be more potent in inhibiting the over all cell proliferation, inducing a significant decrease in cell number starting at concentrations of 30 μM, and further reducing the cell number to 45% of control at concentration of 100 μM. Pamidronate had only a moderate effect on the total cell number (reduction to about 85% of control) at the highest dose (100 μM).

These results suggest that the anti-tumor activity of amino-bisphosphonates on melanoma *in vitro* does not correlate well with the described antiresorptive potency of these compounds *in vivo*. They indicate that zoledronate affects mostly the cell growth, while pamidronate acts rather by inducing cell death. Similar differences in the actions of the two agents have also been reported in most of the studies in breast cancer cells (BOISSIER *et al*., 2000) or prostate cancer (LEE *et al*., 2001). Pamidronate has also been shown to be more effective in inducing cell death than other bisphosphonates with higher antiresorptive potency in macrophages (COXON *et al*., 2000; BENFORD *et a*l., 2001), adenocarcinoma (SURI *et al*., 2001) or prostate cancer (SENERATNE *et al*., 2000). Furthermore, pamidronate and zoledronate have shown similar potency in inhibiting cell proliferation in osteoblasts in a study by Reinholz (REINHOLZ *et al.,* 2000). These data appear to be in contradiction with the finding that the rank order of antiresorptive potency *in vivo* for the amino-bisphosphonates closely matches the order of potency in inhibiting the farnesyl pyrophosphate synthase *in vitro* in osteoclasts and macrophages (DUNFORD et al., 2001),

as well as in inducing apoptosis in osteolcasts (COXON *et al*., 2000; VAN BEEK *et al*., 1999).These discrepancy may find various explanations:

First, although amino-bisphosphonates have been shown to share an inhibitory effect on mevalonate pathway and particularly on farnesyl pyrophosphate synthase, different other enzymes of this pathway, such as squalene synthase (ROGERS *et al*., 2000) or isopentenyl pyrophosphate isomerase (THOMPSON *et al*., 2002; VAN BEEK *et al.,* 1999), may be also selectively influenced by different bisphosphonate derivatives. Second, although the inactivation of prenylated signalling proteins was postulated as main mechanism of action of amino-bisphosphonates, the exact G-proteins inhibited by each bisphosphonates, as well as their expression, their activity and the main cellular processes that are affected by their inhibition within each type of cell are far from being elucidated. In addition to this, besides inhibiting the mevalonate pathway, several other secondary mechanism of action have been proposed for some bisphosphonates such as inhibition of protein tyrosine phosphatases (SCHMIDT *et al*., 1996). The combination of all this factors may therefore account for the reported variance in the effects of bisphosphonates *in vitro*, which appear to be both compound- and cell type- specific.

At last, the lack of correlation between the *in vitro* effects of bisphosphonates and the antiresorptive potency *in vivo* may reflect the role of additional non-cellular factors, that regulate the effect of bisphosphonates on the bone metabolism in the living organism, such as bioavailabilty, affinity to bone, turnover and metabolism within the bone, solubility and cellular uptake.

The apoptotic effect of the two amino-bisphosphonates in melanoma cells was both conentration and time-dependent. A raise in DNA fragmentation could be seen first after 12h of incubation with 100 μM concentration of the compounds, and this effect markedly increased after 24 h incubation. At 48 hours no apoptosis could be measured, but only a high degree of secondary necrosis (data not shown). In contrast to apoptotic stimuli acting through rapid signalling pathways (such as the death receptors- dependent apoptotic pathway), bisphosphonate have been shown to require a certain delay in inducing apoptosis. Latency periods of 12 to 48 h in initiating apotosis by these compounds have also been reported in different other cell systems (JAGDEV *et al*.,2001; FROMIGUE *et al*., 2000; SENERATNE *et al*., 2000) and this is consistent with the postulated inhibitory effect of amino-bisphosphonates on mevalonate pathway. This is, the latency in apoptosis initiation is cell type dependent and may represent the time interval necessary for the depletion of the pre-existing cellular pool of prenylated proteins that maintain cell survival. The turnover rate of the prenylated proteins determines the moment when the

effects of bisphosphonate first become evident. This behaviour of the bisphosphonates is similar with that observed in different cell types with for other inhibitors of the mevalonate pathway such as statins (inhibitors of HMG-CoA reductase) (PADAYATTY *et al*.,1997; PEREZ-SALA *et al*., 1995), confirming, indirectly, once more the involvement of mevalonate pathway inhibition in the cellular effects of amino-bisphosphonates.

To further investigate the time-dependency of the proapoptotic effect of amino-bisphosphonates, A375 cells were exposed for only 6 h to 100 μM pamidronate, followed by additional incubation with drug-free culture medium up to 24 h. This short-time treatment resulted in an almost similar extent of apoptosis as the continuous incubation with the agent over 24 h, demonstrating that short term exposure to bisphosphonates is sufficient to induce apoptosis.

Similar results have been obtained in macrophages (COXON *et al*., 2000), breast cancer (JAGDEV *et al*., 2001) or prostate cancer cells (LEE *et al*., 2001), where exposure to nitrogen-containing-bisphosphonates for as little as 2 h, followed by incubation with culture medium resulted in an apoptotic effect comparable with that of continuous incubation with the drugs. Two main consequences are drawn from this experiment: First, the short time exposure to the agent reflects more accurately the clinical setting where, due to the rapid entrapment of bisphosphonates into bone, the visceral melanoma metastases could probably be exposed to these compounds only for few hours. The high extent of apoptosis measured in melanoma cells after 6 h pulse therapy confirms that an anti-tumor effect of these compounds could also be achieved in clinical situation.

Second, these observations confirm that the delay in apoptosis induction is related to the mechanism of action of amino-bisphosphonates and is not due to the time required for internalization of sufficient amount of drug. This conclusion is also consistent with the observation that fluorescently labelled bisphosphonates are internalized into endocytic vacuoles by J774 and osteoclast *in vitro* within few minutes (CHESTNUT *et al*.,1995).

Mechanism of induction of apoptosis by bisphosphonates

Apoptosis is regulated by a complex interaction between a multitude of signalling factors, in which mitochondria plays a pivotal role; the release in cytosolic compartment of various mitochondrial proteins such as cytochrome c, as response to different apoptotic stimuli, activates the caspases cascade, committing ultimately the cell to apoptotic death. The mitochondrial checkpoint is controlled by different factors, among which the Bcl-2 protein family have an essential role. Bcl-2 protein has been shown to protect cells against the action of various

proapoptotic stimuli, and appears to block apoptosis at mitochondrial level, preventing the release of cytochrome c into cytoplasma. In melanoma, high levels of Bcl-2 have been correlated with resistance against chemotherapy (JANSEN *et al.,*1998). Moreover, raising the ratio between Bcl-2 and proapoptotic members of the Bcl-2 protein family such as Bax has been demonstrated to be an important mechanism of cell survival in different tumors including melanoma (*BOISE et al*., 1993; RAISOVA *et al*., 2001)

The influence of Bcl-2 protein on bisphosphonate-induced apoptosis in melanoma was investigated using melanoma A375/Bcl-2 cells, which stably overexpress the murine Bcl-2 protein, and cells transfected with the empty vector (A375/ pIRES) as controls. In this cell system, overexpression of Bcl-2 had been previously shown to render the cells insensitive to apoptosis induced by death receptors ligand (FasL) or ceramides (RAISOVA *et al*., 2001; RAISOVA *et al*, 2002).

In these experiments, no difference in the extent of apoptosis could be observed between Bcl-2-overexpressing cells and cells transfected with the empty vector, after treatment with 100 μM pamidronate or zoledronate for 24 h. This would suggest that bisphosphonates induce apoptosis in melanoma cells *in vitro* through a pathway that circumvents the mitochondrial checkpoint regulated by Bcl-2. However, in the condition of pulse exposure to pamidronate, apoptosis in A375/Bcl-2 cells exposed for only 6 h to the drug was 270 % of control, compared to 470 % of controls in the same cells continuously incubated with the drug for 24 h. In parallel, in the A375 cells transfected with the empty vector, no difference of apoptotic effect was seen between 6 h pulse therapy and 24h continuous incubation with pamidronate. This difference in apoptosis induction after short-time exposure to bisphosphonates between Bcl-2 overexpressing melanoma cells and the cells transfected with the empty vector only, could indicate that Bcl-2 overexpression can not rescue the cells from apoptosis induced by prolonged treatment with bisphosphonates, but may have, however, some protective effect in the case of short time exposure of cells to the drugs. The functional significance of these observations remains however to be elucidated.

In other cell systems, pamidronate has also been shown to decrease the Bcl-2 expression in a time dependent manner in breast cancer cells (SENERATNE *et al*., 2000). In contrast to our data in melanoma cell lines, in myeloma cells *in vitro*, overexpression of Bcl-2 could block the apoptosis but not the proliferation inhibition induced by zoledronate (APARICIO *et al*., 1998).

The release of cytochrome c from the mitochondria represents a hallmark of the apoptotic pathways with mitochondrial involvement. In cytoplasma, its association with Apaf-1 and pro-

caspase 9, forming the "apoptosome", leads to activation of effector caspases and marks "the point of no return" in cell death. The release of cytochrome c is regulated by the proteins of Bcl-2 family, Bcl-2 and its antiapoptotic relatives (Bcl-X_L etc) blocking the transfer of cytochrome c to cytoplasmic compartment.

In the present study, cytochrome c release has been investigated by analysing the amount of cytochrome c in the cytoplasma of A375/ Bcl-2 and A375/pIRES melanoma cells treated with 100 μM pamidronate, compared to untreated cells as control. A Western blot method was performed, using antibodies against denaturated cytochrome c. As bisphosphonate–induced DNA fragmentation increased between 12 and 24 h after exposure to the drugs, the cytochrome c release was measured at an intermediate time point, at 16 h incubation with pamidronate. In this experiment, no increase in the cytochrome c content of the cytoplasmic fraction could be demonstrated in pamidronate treated cells. A similar result was obtained after 24 h treatment (data not shown). As positive control, cytochrome c release was demonstrated in HL-60 cells treated with etoposide.

The absence of cytochrome c release after pamidronate treatment would be consistent with the failure of Bcl-2 in preventing bisphosphonate-induced apoptosis, these two findings together suggesting that bisphosphonates cause apoptosis by a mechanism which is independent of the mitochondrial checkpoint. At the same time, various reports confirmed that activation of effector caspase 3 is required for the apoptosis induced by bisphosphonates (COXON *et al*., 2000; FROMIGUE *et al*., 2000; BENFORD *et al*.,1999) and a similar observation was made in our group in melanoma cells treated with pamidronate (RIEBLING *et al*., in press). The pathway by which bisphosphonates cause caspase 3 activation in melanoma cells circumventing the mitochondria needs therefore to be further investigated.

Effect of combination of bisphosphonate with standard chemotherapy agents in melanoma cells in vitro

Various chemotherapy regimens are currently used in melanoma for adjuvant or palliative management. It is therefore interesting to study the potential interactions between the classical therapeutical agents of melanoma and the bisphosphonates, as possible candidates for designing new strategy of adjuvant therapy.

DTIC is the most frequently used single agent for therapy of metastatic melanoma. It is an alkylating agent that undergoes activation in the liver via oxidative metabolism. Its action *in vitro* has also been documented (SCHADENDORF *et al*, 1994).

In the present study, the effect of combination between pamidronate 100 μM and DTIC 5μg/ml on DNA fragmentation in cultured melanoma cells was investigated. 5μg/ml was a concentration at which DTIC induced apoptosis but had no cytotoxic effect in the melanoma cells studied (PLEWINSKI, unpublished results). Pamidronate proved to be more efficient than DTIC in inducing DNA fragmentation in both A375 and M186 cells. Moreover the combination of pamidronate with DTIC had approximately the same apoptotic effect as pamidronate alone. It appears thus that no synergistic or additive effect exist between DTIC and bisphosphonates *in vitro*. However, it would be important to explore in addition the possible concentration- and/or temporal relationships between the two drugs. The effect of combinations between bisphosphonates and other chemoterapeutical agents used in melanoma, should also be investigated in further studies, especially since synergistic effects between bisphosphonates and different other antineoplastic agents such as paclitaxel or dexamethasone have been reported in breast cancer (JAGDEV *et al*., 2001) and myeloma cells (TASSONE *et al*., 2000).

Effects of bisphosphonates on the cell cycle progression in melanoma cell lines in vitro

Beside their ability to induce apoptosis, the two amino- containing bisphosphonates caused also alteration in the progression of melanoma cells through the phases of the cell cycle. DNA content by means of flowcytometry revealed that both pamidronate and zoledronate determined accumulation of cells in the S phase of the cycle in the two melanoma cell lines studied, with corresponding decrease in the number of cells in G1 and G2/M phases. This phenomenon could reflect a slowing of progression through S phase or a block before the entry in G2/M phase. This effect was dose-dependent and more marked for zoledronate, which could induce cell cycle changes beginning at concentration of 30 μM. Pamidronate's effects on cell cycle were first visible at 50 μM and matched those of zoledronate at the dose of 100 μM.

These alterations in cell cycle could explain at least in part the antiproliferative activity of bisphosphonates in melanoma cells. Although pamidronate was more active in inducing apoptotic cell death, zoledronate had a stronger effect upon the total tumor growth, as reflected in the study of cell number and of cell cycle progression.

Consistent with the lack of activity of clodronate on cell proliferation and apoptosis in the studied melanoma cells, this compound also failed to significantly alter the cell cycle progression. A slightly different pattern however, with a modest increase in the G1 population of cells accompanied by a corresponding decrease of cells in S and G2/ M phase, was observed at 1000 μM clodronate.

The mechanism of these changes is not clear. Similar delay in the S phase progression has been documented in myeloma (APARICIO *et al*., 1998), prostate cancer (LEE *et a*l., 2001) or keratinocytes (RESZKA *et al*., 2001) treated with amino-bisphosphonates, and it could be related to the inhibition of prenylation of small GTPases.

The progression of the cells through the cell cycle is controlled by positive and negative regulatory proteins. Cyclin-dependent kinases (CDKs) (CDK2, CDK4, CDK4) act as positive regulators. In complexes with cyclins (D, E or A-type) they become active and cause phosphorylation and inactivation of the retinoblastoma (Rb) tumor suppressor protein. Phosphorylated Rb releases the bounf E2F, which is then allowed to activate the transcription of genes necessary for progression through the cell cycle. The regulatory Rb pathway is inhibited by CDK inhibitors (CKI) of INK (p16 ^{Ink4a}, p15^{Ink4b}) and Cip/Kip families (p21^{Cip1}, p27^{Kip1}). Different cyclin-CDK complexes seem to be preferentially active in different phases of the cell cycle. Thus, initiation and progression through the G1 phase is controlled mostly by D type cyclins (D1, D2, D3) which complex CDK 4 or CDK 6. Further, in late G1 and S phase, cyclin E /CDK2 complexes become the driving force in advancing the cell cycle.

Both Ras and Rho proteins are known as important regulators of the cell cycle. Activated Ras and Rho promote exit from the G0, passage through G1 and entry in S phase by controlling the expression and function of cyclin D1, p21^{Cip1}, and p27^{Kip1}. Cyclin D1 is a critical target of the mitogenic Ras signalling cascade. The Rho proteins, in addition, promote p27^{Kip1} degradation and activation of cyclin E/CDK2 complex, which regulate mainly the S phase.

Therefore, inactivation of Ras or Rho proteins via inhibition of prenylation by bisphosphonates could explain the alterations of the cell cycle observed after treatment with these compounds. It would also be consistent with the observation that other inhibitors of the mevalonate pathway, such as statins, also induce comparable cell cycle changes (VOGT *et al.*, 1997; NADERI *et al*., 1999). Accumulation of cells in S phase could suggest that regulators of synthesis phase, such as geranylgreanylated proteins Rho, rather than the farnesylated Ras are the main target of the inhibitory action of bisphosphonates.

However, the time-dependency of these effects of bisphosphonate on cell cycle should be also explored . It would be thus important to establish if the S phase block by bisophosphonates is reversible. As these compounds inhibited but did not abolish the cell proliferation, it is likely that this accumulation of cells in S phase reflect only a slowing of the progression through the S phase than S arrest. A second point of investigation would be the relationship between the cell cycle alteration and the apoptosis induced by bisphosphonates. For example, it may be possible

that the cells abnormally blocked or slowed in S phase are the ones who are sensitive to induction of apoptosis by bisphosphonates, or by other concurrent pro-apoptotic stimuli. The accumulation of cells in the vulnerable S phase could also enhance the effect of antineoplasic drugs acting in this phase.

These findings indicate that bisphosphonates are able to counter tumor growth and survival by interfering with the cellular processes at different levels. Cell cycle alteration, activation of apoptosis and cytostasis may grant for their potential use for designing new strategy in the adjuvant tumor therapy, including that of highly resistant metastatic melanoma. The currently available bisphosphonates however represent only the basis for development of new compounds with different pharmacologycal and pharmacodynamic properties as well as with higher specificity of cellular effects that may reach the desired efficacy also in the treatment of visceral metastases of human tumors.

SUMMARY

This study compared for the first time the *in vitro* effects of different bisphosphonates on melanoma cell lines. Three compounds with different mechanism of action and different antiresorptive potency were analysed: a non-amino-bisphosphonate (clodronate) and two amino-bisphosphonates, pamidronate and zoledronate, which is the most potent antiresorptive agent known to date. Their effects on cell proliferation, induction of apoptosis and cell cycle progression in the melanoma cell lines A375 and M186 and in the Bcl-2 overexpressing cell line A375/ Bcl-2 have been investigated in detail.

In this experimental model, it could be shown that the nitrogen-containing bisphosphonates inhibit cell proliferation, induce apoptosis and alter cell cycle progression causing accumulation of cells in the S phase of the cycle. These effects were dose dependent, and were achieved at concentrations ranging from 10 to 100 μM. The antiproliferative and proapoptotic activity of the two compounds *in vitro* did not correlate well with their known antiresorptive potency *in vivo*. However, despite a stronger effect of pamidronate in inducing apoptosis, the more potent antiresorptive zoledronate was more effective in inhibiting cell proliferation and altering cell cycle progression, showing a stronger influence on the overall tumor growth.

Apoptosis induced by the amino-bisphosphonates was dose and time dependent and acute exposure to pamidronate over 6h was sufficient to induce apoptosis. The pro-apoptotic effect of pamidronate and zoledronate was not inhibited by overexpression of Bcl-2 protein and did not appear to involve of cytochrome c release from the mitochondria, suggesting that these compounds may stimulate a mitochondria-independent pathway for inducing apoptotosis.

In contrast, the non-amino-bishophonate clodronate had no effect on cell proliferation, apoptosis induction or cell cycle progression in melanoma cell lines, even at high concentrations. This finding further confirms differences in the intracellular mechanism of action between the two types of bisphosphonates: the amino bisphosphonate (supposed to act by inhibition of protein prenylation) and the non-amino-bisphosphonates (who are reportedly metabolised to toxic ATP analogues). The latter mechanism seems to have no functional significance in melanoma cell growth.

In conclusion, amino-bisphosphonates manifest a direct antitumoral effect on melanoma cells *in vitro*, and may thus represent a promising novel class of agents for the treatment/prevention of melanoma metastasis. Further studies are required, in order to describe the exact mechanism of action of these compounds, their most effective structures and ultimately their potential place in new strategies for adjuvant therapy schedules in malignant melanoma.