INTRODUCTION

1.1. STRUCTURE, PHARMACOLOGY AND MECHANISMS OF ACTION OF BISPHOSPHONATES

Bisphosphonates are a family of synthetic analogues of pyrophosphate (PPi) which are currently widely used in the treatment of metabolic bone disorders such as osteoporosis, Paget's diseases of bone and tumor-related osteolysis. Although they have been used in the clinical practice for more than 30 years, their mechanism of action only begins to be unraveled, and recent studies indicate that beside their ability to inhibit bone resorption, bisphosphonates also have a direct antitumoral effect, interfering with cell proliferation, cell survival and metastasing in different types of human tumors.

1.1.1. Structure

Structurally, bisphosphonates are characterized by a phosphorus-carbon-phosphorus backbone (P-C-P) which, unlike the unstable pyrophosphate is highly resistant to hydrolysis under acidic condition or by pyrophosphatases. Different members of this drug class differ from each other through the side chains (R^1 and R^2) attached to the geminal carbon atom (Fig.1).

Like PPi, bisphosphonates form a three-dimensional structure capable of binding bivalent metal ions such as Ca^{2+}, Mg^{2+}, and Fe^{2+} in a bidentate manner, by coordination of one oxygen atom from each phosphonate group with the divalent cation. The affinity for calcium can be further increased if one of the side chains (R^1) is a hydroxyl (-OH) or a primary amino ($-NH_2$) group, because this allows the formation of a tridentate conformation that is able to bind Ca^{2+} more effectively (JUNG *et al,* 1973).

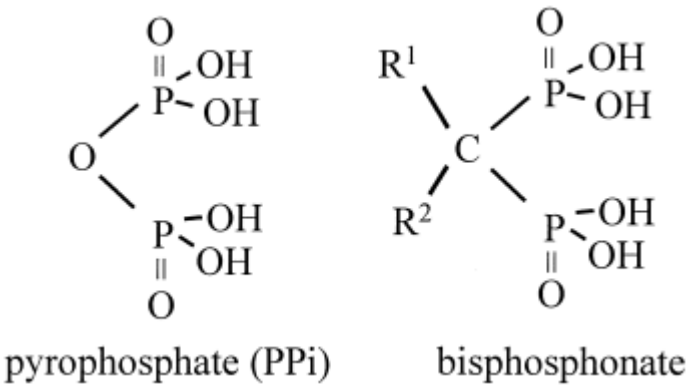

Figure 1. Chemical structures of pyrophosphate and bisphosphonates

The high affinity of bisphosphonates for Ca^{2+} ions is the basis of the bone-targeting property of these compounds, and leads to rapid clearance of the drugs from the circulation and localization to hydroxyapatite bone mineral surfaces. Moreover, the ability to adsorb to bone mineral *in vivo*, electively delivers bisphosphonates to sites of active bone remodeling, where they are potent inhibitors of bone resorption mediated by osteoclasts. This phenomenon explains the wide use of bisphosphonates in the treatment of diseases involving excessive osteoclast activity.

The geminal carbon of the bisphosphonate can covalently bind carbon, oxygen, halogen, sulfur, or nitrogen atoms, giving thus raise to a huge range of possible structures. From these, clinically used in present are the compounds**:** clodronate, alendronate, pamidronate, ibandronate and risedronate, and recently zoledronate, which differ greatly in their antiresorptive potency (Table 1**)**

Table 1. Relative antiresorptive potency of bisphosphonates *in vitro* (PATERSON, 2000)

Bisphosphonates	**Relative antiresorptive potency *(in vitro)***
etidronate	1
clodronate	10
pamidronate	100
risedronate	1000
alendronate	10,000
ibandronate	50,000
zoledronate	100,000

The structure and the length of the R^2 chain appears to be the main factor deciding the anti- resorptive potency and also the mechanism of action of different bisphosphonate compounds. The least potent antiresorptive agents and the first introduced in clinical practice were those which resemble the most the endogenous PPi: compounds such as clodronate or etidronate have simple structure side chains, e.g. $-CH_3$ and -OH.

The antiresorptive potency can be improved by increasing the length of R^2 side chain and by inserting an amino group in its structure. Bisphosphonates with an amino-alkyl group in the R^2 chain position, the so-called amino-bisphosphonates such as pamidronate and alendronate are up to 1,000 times more potent bone protectors *in vivo* as etidronate. The optimal length of the amino-alkyl R^2 side chain appears to be of 4 C-atoms (as in alendronate).

The amino group in the R^2 side chain has a particular importance in assessing the potency of a bisphosphonate compound. Thus, bisphosphonates containing a secondary amino group are more potent as the primary amino containing compounds. Furthermore, bisphosphonates containing a tertiary nitrogen within a ring structure, such as zoledronate and risedronate appear to be the most potent antiresorptive agents discovered to date, with a 10,000 times stronger bone protective effect *in vivo* as etidronate (SIETSEMA *et al*.,1989; GREEN *et al*., 1994) (Fig.2). However, transforming the tertiary nitrogen atom of the pyridyl ring of risedronate in a quaternary pyridinium nitrogen by alkylation does not further increase the antiresorptive potency (ROGERS *et al.*, 2000).

Beside R^2 chain, the phosphonate groups and the R^1 also influence the antiresorptive efficiency of bisphosphonates. All currently clinically used bisphosphonates (with the exception of clodronate) contain a -H or -OH group in the R^1 position, which increases the affinity of these compounds to bone. The P-C-P structure itself, also appears to be important not only for targeting the bisphosphonates to bone mineral, but also for the interaction with the molecular target of the drug (EBETINO *et al,* 1996).

These particularities of the structure-activity relation of bisphosphonates may be useful in the identification of new compounds with similar molecular effects, but with less affinity for bone mineral and thus with possibly greater systemic effects.

1.1.2. Mechanism of action

It was noted, early in the development of the bisphosphonates class, that the antiresorptive potency of different compounds did not correlate to their affinity to Ca^{++} and bone; this suggested that bisphosphonates may inhibit bone resorption through a cellular effect on bone resorbing cells, rather than by protecting hydroxyapatite from dissolution through a mere physicochemical mechanism.

Different authors have shown that bisphosphonates can enter cells of macrophage lineage and can be then found in the mitochondria and others organelles of the cells. *(*FELIX *et al.,* 1984*)*. Studies with slime mold amoebae, the growth of which is inhibited by bisphosphonates, have also demonstrated that cellular uptake occurs by fluid-phase endocytosis and that the mechanism of growth inhibition is intracellular rather than extracellular (ROGERS *et al.,* 1997). Taken together, these observations suggest that bisphosphonates can be internalized by cells by pinocytosis and indicate a cellular mechanism for their antiresorptive effect.

It is now generally accepted that the protective effect of bisphosphonates on bone is mostly due to their action on the osteoclasts. Numerous studies have shown that bisphosphonates are able to inhibit osteoclast recruitment, prevent cell growth and induce apoptosis in osteoclasts, disrupt the cytoskeleton organization, reduce the osteoclast adhesion, and inhibit osteoclasts' resorptive activity. Moreover, similar inhibitory effects of bisphosphonates on cell growth and survival, on cell adhesion and motility, have been also demonstrated in osteoblasts, macrophages and, recently, in tumor cells.

The intimate mechanisms responsible for these cellular effects only begin to be elucidated. Recent studies have found that nitrogen-containing bisphosphonates can specifically inhibit enzymes of the intracellular mevalonate pathway, which is required for the synthesis of cholesterol and isoprenoid lipids such as farnesyldiphosphate (FPP) and geranylgeranyldiphosphate (GGPP) (Fig.3). (LUCKMAN *et al.,* 1998; BENFORD *et al.,* 1999; VAN BEEK *et al.,* 1999) The FPP and GGPP residues are necessary for the post-translational lipid modification (prenylation) of different signaling proteins such as the small GTPases.

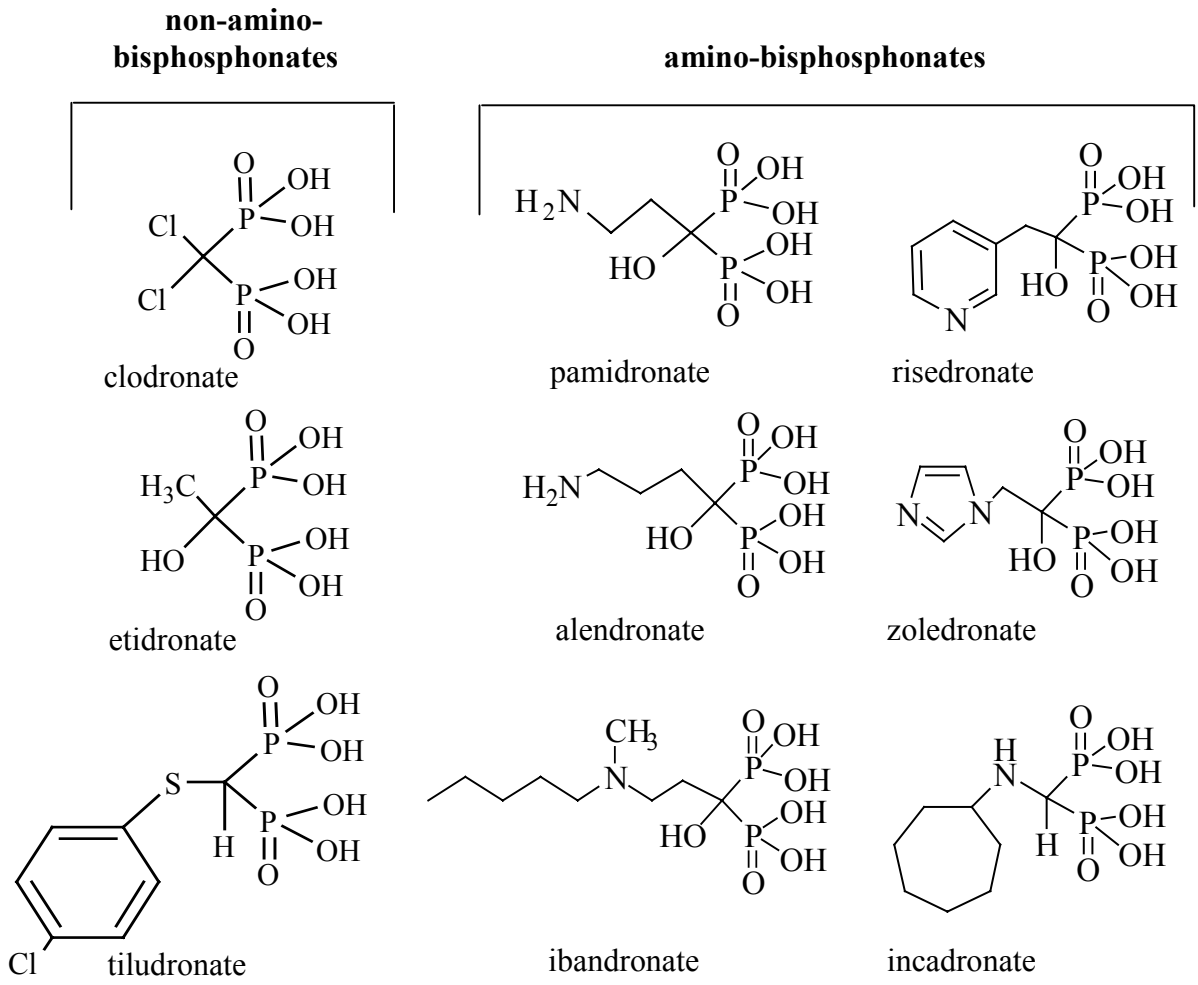

Figure 2. Comparative structure of different bisphosphonates

For these proteins the prenyl groups act as membrane anchors, being essential for their activation and interactions with other proteins. The small GTPases, such as Ras, Rho, Rac and Rab are important signaling proteins, being involved in a multitude of cellular processes including the control of cell morphology, cytoskeleton organization, membrane trafficking, transcriptional regulation, cell growth and apoptosis (AZNAR and LACAL, 2001*)*. Their inactivation, via inhibition of prenylation could therefore explain at least in part the cellular effects of bisphosphonates. Since the cellular effects of amino-bisphosphonates can be entirely overcome by geranylgeraniol supplementation, and only partially by farnesol addition, it is likely that the depletion of geranylgeranylated proteins is the major molecular mechanism of action of these drugs. The precise signaling pathways, involving prenylated small GTPases, that are affected by bisphosphonates remain to be determined.

The exact enzymes of the mevalonate pathway inhibited by bisphosphonates are being currently identified. Incadronate and ibandronate are known as inhibitors of squalene synthase (AMIN *et al.,* 1992). Alendronate, pamidronate, risedronate and ibandronate have been shown to inhibit dose-dependently farnesyl pyrophosphate-synthase and/ or isopentenyl pyrophosphate isomerase (VAN BEEK *et al*., 1999). The bisphosphonate group of the bisphosphonates appear to fit into the diphosphate binding site of the farnesyl pyrophosphate-synthase, explaining the importance of changes in the bisphosphonate group structure for the activity and potency of different bisphosphonates (LUCKMAN *et al.,*1998). Furthermore the nitrogen in the R^2 side chain of bisphosphonates has been shown to act as a carbocation transition state analogue (MARTIN *et al.,* 1999) which is stabilized by oxygen atoms in the active site cleft of farnesyl pyrophosphate-synthase. The length and orientation of the R^2 chain of bisphosphonates affects the interaction of the nitrogen with amino-acid residues in the active site cleft hence explaining the role of the structure or conformation of the R^2 side chain in the potency of bisphosphonates (SIETSEMA *et al*.,1989; ROGERS *et al*., 1995; VAN BEEK *et al*., 1994). All these studies indicate farnesyl pyrophosphate synthase as a major pharmacological target of the bisphosphonates. However, as several other enzymes of the mevalonate pathway use an isoprenoid diphosphate as substrate and thus may have a similar substrate-binding site, it is possible that bisphosphonates also interact with other enzymes of the mevalonate pathway as well.

In contrast to the nitrogen containing compounds, bisphosphonates who lack an amino group in the R^2 side chain, such as clodronate and etidronate, appear to have no effect on the mevalonate pathway. Several studies have shown that these non-amino bisphosphonates, which closely resemble pyrophosphate, can be metabolically incorporated into non hydrolysable, methylene containing analogues of adenosine triphosphate (ATP) (ROGERS *et al.,* 1996). These inactive metabolites of adenosine-P-P-C-P type could then compete with ATP in enzymatic reactions, disturbing the energy production of the cell and thereby explaining the growth inhibitory and cytotoxic effects of these bisphosphonates.

Beside these two main mechanisms of action, different studies suggest additional molecular effects of the bisphosphonates, such as inhibition of the proton-change-ATPase (DAVID *et al.,* 1995*)* and inhibition of protein tyrosine phosphatases (SCHMIDT *et al*., 1996). Thus, although bisphosphonates are in present usually divided in two main pharmacological classes (those who inhibit protein prenylation and those who are metabolized to ATP analogues), it is likely that within each class, individual compounds may also act through different pathways, explaining their complex cellular effects.

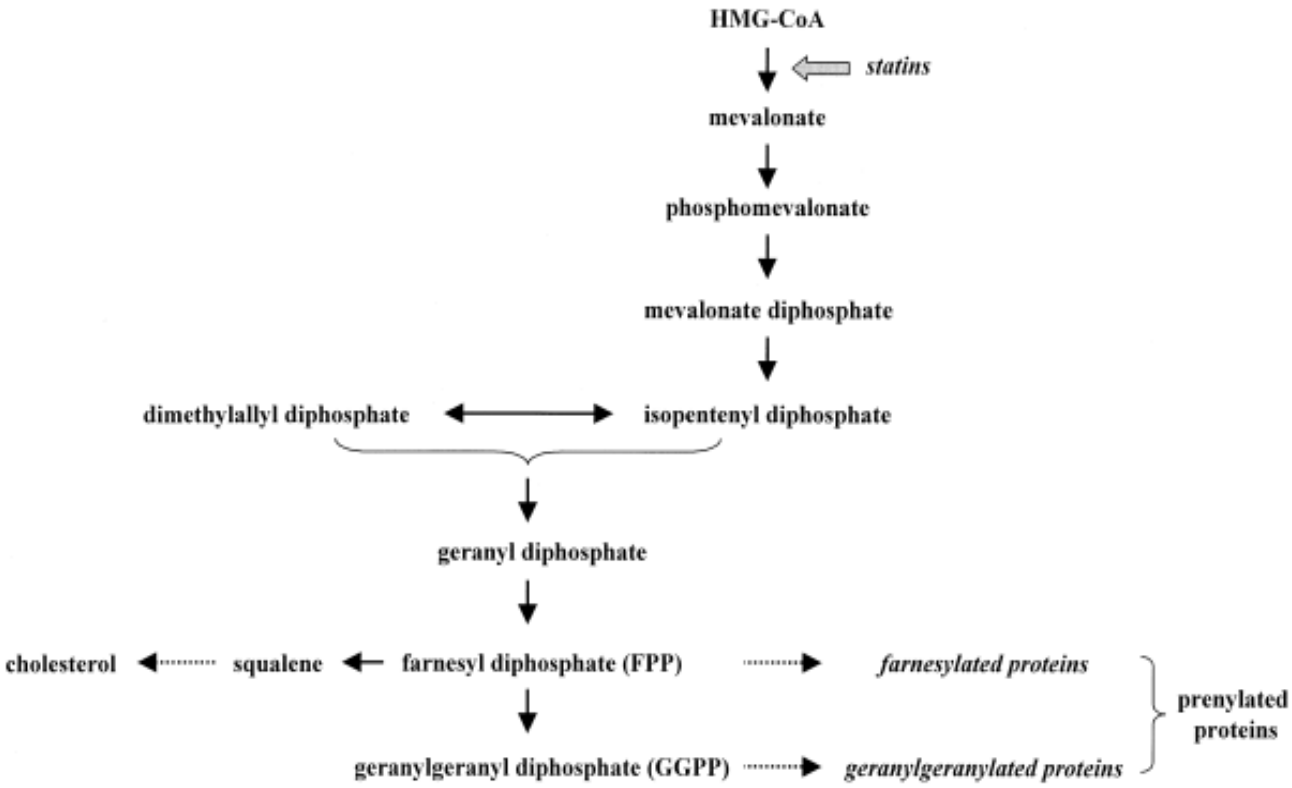

Figure 3. Mevalonate pathway

1.1.3. Pharmacology

Bisphosphonates have unique, non-linear pharmacokinetic characteristics that result from their highly polar structure.

Oral bisphosphonates are poorly absorbed, mainly from the upper gastrointestinal tract (HOFFMAN *et al.,* 2001) with 1 % or less of the administered dose being taken up. Intestinal absorption is almost entirely prevented by the presence of food containing calcium or other divalent ions, which chelate the bisphosphonate. On the other hand, the administration of EDTA, which chelates calcium, increases the absorption of bisphosphonates (JANER *et al*., 1991). Absorption is also increased by high intraluminal pH, e.g. in H_2 receptor antagonist therapy (GERTZ *et al.,* 1995).

Following intravenous infusion bisphosphonates are rapidly cleared from plasma, mostly through renal excretion and bone uptake and entrapment. The bisphosphonates are generally not metabolized in the organism and are eliminated in urine as the parent compound being therefore regarded as ”hard drugs” (LIN *et al.,*1996).

The resorption surface of the bone bears the highest concentration of the drug in organism. In the bone, the ability of bisphosphonates to chelate Ca^{++} ions is reduced at low pH due to protonation of the phosphonate groups (EBETINO *et al.,* 1998). Hence, in the acid environment of resorption lacunae, bisphosphonates may be released from bone surfaces, causing high local concentrations of drug in solution or calcium salts. This may explain the selective effects of bisphosphonates on bone, in the conditions of low serum- and soft tissues levels. Bisphosphonates which become buried in bone through new bone substance formation are considered inactive, the recent studies suggesting that only bisphosphonates bound to bone surface possess biologic activity (RODAN *et al.,* 1993).

The bioavailability of bisphosphonates in non-osseous tissues follows a complex dynamic, and seems to be dependent on the administration route of the drugs. In a study of Hofmannand coworkers, the drug concentrations in soft tissues were found to be different following oral administration, continuous perfusion, iso-osmotic or hypo-osmotic bolus i.v. administration respectively. Furthermore, the bioavailability was influenced by the structure and physico-chemical properties of each bisphosphonate derivative.

Oral administration seems to result in general in higher tissue concentration, as well as in a longer elimination time than the i.v. route of administration (HOFFMAN *et al.,* 2001). For pamidronate, the same study showed higher tissue levels after bolus i.v. administration as after continuous perfusion, and hypotonic i.v. bolus administration resulted in higher liver and spleen drug concentration as the isotonic mode of administration. In contrast, other bisphosphonate derivatives, with different protein binding properties and solubility pattern, showed an inverse tissue distribution pattern.

The mode of administration also influences the adverse effects of the bisphosphonates therapy. Oral administration may be accompanied by gastrointestinal complains, such as nausea, vomiting and pain, as well as oesophagitis (DE GROEN *et al*., 1996; LUFKIN *et al.,* 1994*)* and gastric ulcer (GRAHAM *et al*., 1999). These effects seem to be stronger with nitrogen containing bisphosphonates as with clodronate or etidronate, and could be related to the cellular damage or apoptosis of the GI epithelial cells. In i.v. administration, the main adverse effects are reactions at injection site and flu-like syndrome. Higher doses may occasionally result in severe disturbance of kidney function. Overall however, the tolerability of bisphosphonates is very good and these compounds can be safely administered even for longer periods (ALI *et al.,* 2001).

At the present time there is no agreement concerning the most appropriate route of administration of bisphosphonates, and the disease's clinical setting, patient compliance, associated medication and ultimately individual drug characteristics are the decisive factors for the therapeutic regimen, until information from long-term and larger clinical trials becomes available.

1.2 USE OF BISPHOSPHONATES IN TUMOR THERAPY

Due to their antiresorptive and bone protecting activity, bisphosphonates came in intensive use in the clinical management of metastasis induced osteolysis. In the last decades, numerous clinical trials have demonstrated the beneficial effect of bisphosphonates on metastasis- related fractures, pain, and hypercalcemia, which matches the antiresorptive potency of various compounds. Recent work however, focusing on the cellular and molecular mechanisms of action of the bisphosphonates, reveals their potential role in disrupting the complex interactions between tumor cells and bone microenvironment, and offers new insights for their possible direct antitumoral effects.

In present, the use of bisphosphonates is recommended only as palliation, in addition to classical anti-tumor therapy, in metastatic malignancy with proven bone involvement (HILLNER *et al.,* 2000). Bisphosphonates are not, for the time being, approved as adjuvant therapy of malignancy.

The bisphosphonates regimens currently in clinical use in metastatic bone disease are: clodronate p.o. 1600-2400 mg/d, clodronate i.v (1600 mg/d) and pamidronate i.v., 90 mg in 2 h slow perfusion, every 3-4 weeks. Pamidronate i.v. is the only bisphosphonate approved by the FDA for metastatic bone disease. Newer bisphosphonates recently available on the market are zoledronate in dosage of 4 mg i.v. over 15 min (phase III clinical trial ongoing in 2001) (COLEMAN *et al*., 2001) and ibandronate in oral or i.v. formulations, and are still under evaluation.

1.2.1 *In vitro* studies of anti-tumor effect of bisphosphonates

In the last years, a continuously growing amount of experimental evidence has accumulated concerning the ability of bisphosphonates to reduce cell viability in different types of human tumor cell lines. Different bisphosphonates have been shown to inhibit cell growth and/or induce apoptosis *in vitro* in myeloma (APARICIO *et al.,* 1998; SHIPMAN *et al.,* 1998; TASSONE *et al.,* 2000), breast and prostate carcinoma (LEE *et al.,* 2001; FROMIGUE *et al.,* 2000; SENERATNE *et al.,* 2000, JAGDEV *et al.,* 2000), osteosarcoma (SONNEMANN *et al*., 2001) and colon carcinoma (SURI *et al.,* 2001). The apoptosis induction in tumor cells appears to follow the two main mechanisms of action described for bisphosphonates: inhibition of mevalonate pathway and production of toxic ATP analogues. The intensity of proapoptotic and antiproliferative effects of bisphosphonates does not always correlate with their relative antiresorptive potency, and seem to be cell type specific. The inhibition of tumor cell growth could be further related to the effect of bisphosphonates on the cell cycle, as several studies have shown that bisphosphonates can induce cell cycle alteration in tumor cells, with delay in cell cycle progression and accumulation of cells in S phase (APARICIO *et al*.,1998; RESZKA *et al*., 2001).

Beside their cytotoxic activity, bisphosphonates can also alter the adhesion and motility of malignant cells .Van Pluijm et al demonstrated that the more potent bisphosphonates can inhibit the adhesion of breast carcinoma cells to rat bone matrices, although no effect was seen with etidronate and clodronate in this system (VAN PLUIJM *et al.,*1996). This effect was confirmed by Boissier and Magneto in both prostate and breast cancer cells (BOISSIER *et al*., 1997) and a further study also showed that bisphosphonates can inhibit breast cancer cells invasion *in vitro (*BOISSIER *et al.,* 2000). Inhibition and down-regulation of metalloproteinases (TERONEN *et al.,*1999*;* BOISSIER *et al.,* 2000*)*, also influencing the invasive properties of malignant cells, is another effect of bisphosphonates demonstrated in tumor cells *in vitro* .

At last, bisphosphonates were shown to enhance *in vitro* the antitumor activity of various established chemotherapeutical agents such as paclitaxel (JAGDEV *et al.,* 2001) in breast cancer or dexamethasone in myeloma cell lines (TASSONE *et al.,* 2000).

1.2.2 Antitumor effects of bisphosphonates in animal models

Table 2 lists some of the preclinical studies performed with bisphosphonates in animal models of bone metastasis (MUNDY 2001).

Risedronate, at dose of 4 µg/d was shown to block bone resorption, reduce the number of new metastases and inhibit the progression of existing lesions in a nude mouse model (SASAKI *et al.,* 1995). Tumor volume was also notably reduced in risedronate treated mice. Similar effects of risedronate were observed in rat mammary adenocarcinoma (HALL *et al.,* 1994).

Table 2. Preclinical studies of bisphosphonates in animal models of bone metastasis

Study	*Treatment*	*Animal Model*	*Dosing regimen*	*Bone lesions/ Osteolysis*	*Tumor in bone*	*Extra-osseous Tumor*
Sasaki *et al* 1995	risedronate	Nude mouse, breast cancer (MDA-231)	4 µg/d	Decreased	Dereased	Increased
Sasaki *et al* 1998	YH529	Nude mouse, breast cancer (MDA-231)	0.2, 2.0, 20 µg/d	Decreased	Decreased	Increased with 0.2 and 2.0, decreased with 20 µg/d
Krempien *et al* 1993	clodronate	Rat model, Walker 256 carcino-sarcoma	30 mg/d x 5 or 2.5-5.0 mg/d x 21	Decreased	No notable effect	No effect
Kostenuik *et al* 1993	pamidronate	Rat model, Walker 256 carcino-sarcoma	0.5 mg/kg/d x 7	Decreased	Increased	No effect
Yoneda *et al* 1997	ibandronate	Nude mouse, breast cancer	4 mg/d	Decreased	Decreased	Micro-metastases present; no effect on survival

Another experimental bisphosphonate, YH529, showed a similar effect on prevention and treatment of bone metastases in nude mouse model (SASAKI *et al*., 1998) and prophylactic s.c. injections of clodronate inhibited osteolysis in the experimental rat model using direct intraosseous implantation of the Walker carcinosarcoma 256B cell line.

In a further syngeneic mouse model pamidronate in continuous injection was showed to inhibit the intraperitoneal growth of murine myeloma cells and to reduce the tumor weight by more than 50 % compared with the control (MULLER *et al.,* 1996). In contrast to these results, Kostenuik and coworkers found that skeletal tumor burden was increased after pamidronate treatment of rats with metastatic Walker 256 cancer cell tumors (KOSTENUIK *et al*., 1993). In another study of Shipman, the potent bisphosphonate ibandronate failed to induce myeloma cell apoptosis or to reduce tumor burden in the 5T2MM murine model of multiple myeloma. (SHIPMAN *et al*., 2000). These results underline the fact that dose selection, tumor type and other factors need to be carefully assessed for each bisphosphonate before entering clinical trials.

Furthermore, at the doses and regimens used so far in animal models, the antitumoral effect of bisphosphonates seems to be limited to bone, with little impact on the nonosseous metastases. This could indicate that the interference of bisphosphonates in the communication between osteoclasts, bone- and malignant cells, is responsible in fact for the beneficial local effect of bisphosphonates. It is also possible that the high local concentration of bisphosphonates in bone play an important role in activity of these drugs on bone tumor burden.

1.2.3 Clinical trials

At present bisphosphonates are not used as antitumor agents, but rather as palliative treatment for the skeletal complication of bone metastases. Improvements in survival time of certain subgroups of patients have been found in several phase III studies with bisphosphonates such as pamidronate *(*BERENSON *et al.,* 1998) or ibandronate *(*DIEL *et al.,* 1999). However, these studies were not targeted to detect survival as a primary or secondary outcome measure, and their results can therefore serve only as orientation for further research.

To date, only 3 major trials have examined the effect of bisphosphonates (clodronate) on survival as primary efficiency parameter in patients with metastasing breast cancer (Table 3) (TRIPATHY*,* 2001).

Table 3. Bisphosphonates as adjuvant therapy: clinical trials

Study	*No. Patients*	*Bone metastases clodronate vs. placebo (2-5 y follow-up)*	*Disease-free state (%)*	*Overall survival (%)*
Diel et al	302	8 % vs 17 %	87 % vs 71 %	96% vs 85 %
Powles et al	1 079	5.2 % vs. 8.1 % (P=0.054)	___	NS
Saarto et al	299	___	52 % vs. 69 %	86 % vs. 81 %

The first, an open label controlled trial by Diel and coworkers, analyzing 302 patients with breast cancer over 5 years, showed an overall increase in the disease free and overall survival time in bisphosphonates treated patients (clodronate 1600 mg/d), as well as a reduction in development of new osseous and tissue metastases.

The Powles study reported also a significant reduction of osseous metastases after treatment with clodronate for 2 years, but the disease free interval and overall survival were unchanged in comparison with the placebo treated group.

In contrast, the third open label study conducted in Finland, (SAARTO *et al.,* 2001) in a 5 years follow-up, showed no reduction in number of osseous metastases in 299 breast cancer patients with lymph node metastasis. Moreover, it reported a deleterious effect on relapse rates of non osseous metastases and a deterioration in overall survival after 3 years treatment with oral clodronate.

These contradictory results reflect mostly the methodological differences between different studies, such as diversity in inclusion criteria, number of patients, and assessment techniques. However, these first clinical data, together with the promising results from the preclinical studies on one side, and the well proven benefit in the palliative care and low toxicity of the bisphosphonates on the other, justify further investigation of a possible role of bisphosphonates as adjuvant therapy. Well designed, large clinical trials are imperatively required in order to elucidate the true benefits of these compounds in cancer therapy and to further describe the most effective administration route and therapeutic regimens.

1.3 MELANOMA: CLINICAL COURSE, PROGNOSTIC PARAMETERS, THERAPEUTIC MEASURES

Melanoma (MM) is a highly malignant tumor originating in the pigment producing cells of the skin, the melanocytes. Its incidence and mortality has continuously increased over the last decades, and its propensity to metastasize and its resistance to therapy in later stages make it the most aggressive skin cancer.

In Europe, the incidence of melanoma is in present about 10-15: 100, 000 inhabitants. In US the risk of developing melanoma for persons born in 2000 is estimated at 1:75, in comparison to 1:250 in 1980.

Melanoma affects mostly young adults, with mean age of incidence of 52 years old. White caucasians are at highest risk, while melanoma in black people is rare. In Europe women seem to be more affected as men. Melanoma can develop in any part of the body, with a predilection for face and legs in women, and for upper back in men. Melanoma of mucosae and of retina account for under 10 % of all cases.

The etiology of melanoma remains unknown, being probably the result of interaction between environmental factors and a genetic predisposition. The main risk factors known to date are: 1) strong intermittent sun exposure leading to sunburns on unadapted skin, especially in childhood; 2) light complexion (fair hair, light skin color, skin types I and II); 3) many naevi or dysplastic naevi ; 4) family history of melanoma.

Familial melanoma may occur in 5-10 % of the cases. 30 % of melanoma develop in association with pre-existent pigment lesions.

1.3.1. Clinical course

Clinically, malignant melanoma appear as dark brown to black skin tumors, which typically meet the ABCDE criteria: ***A***symmetry, irregular ***B***orders, variable ***C***olor, ***D***iameter of more than 5 mm and ***E***levation. Some authors propose the ***E***volution, i.e. any change in lesion's shape, dimension, color, or appearance, as fifth criterion for early melanoma diagnosis.

Four main types of malignant melanoma are described, based on clinical and histological criteria:

1. Nodular malignant melanoma (NMM)

This form of malignant melanoma occurs mostly in 5th and 6th life decades, and it shows an early vertical growth with rapid invasion of dermis. NMM appears as a dark, blue-black nodule or raised plaque relatively sharply demarcated from the normal skin. Sometimes a pink-gray color can be observed. It can develop on unaffected skin or in association with an existing pigment lesion and its history is usually short (months). Election sites are legs in women and upper back in men. Its evolution is relatively fast, in most of cases at diagnosis time the tumor is already invasive, and hence its prognostic is poor.

2. Superficially spreading malignant melanoma (SSM)

This is the most frequent form of MM in white persons. It presents as a sharply demarcated, round-ovalar plaque, 2-5 cm in diameter with round or polycyclic borders. The margins are usually elevated and color varies greatly, from dark brown and black to shades of gray, pink or white (in regression areas). The lesion is at the beginning flat, and grows slowly horizontally; subsequently its surface becomes irregular as circumscribed infiltrated papules or nodules develop, signaling vertical growth.

The prognosis is good in early phases (horizontal growth). When vertical growth and dermal invasion occurs, the metastasing risk is significantly high.

3. Lentigo maligna melanoma (LMM)

This clinical form accounts for about 10 % of the MM cases. LMM develop mostly in elderly, on sun-exposed areas, superimposed on a preexistent lentigo maligna lesion. A lentigo maligna can growth for years or even decades before developing a malignant character. LMM presents like a large macular lesion, with colors varying from light to dark brown and black, and circumscribed dark nodules on its surface. The elevated areas represent regions of vertical growth and invasion. Gray pink or white areas occur and are interpreted as signs of regression.

The prognostic of this type of melanoma is more favorable, as the vertical growth occurs only late.

4. Acro-lentiginous malignant melanoma (ALM)

This type of melanoma is rare in the white population (5 %), but it is the principal form in blacks (50 % of melanoma in American and African black population). ALM occurs primarily on palms and soles as well as on toes and fingers. It can develop peri -or subungual with hyperpigmentation or destruction of nail plate. Melanoma of the mucosae falls in this same category.

Clinically it presents as maculae or plaques, with color varying from brown to black. After an initial horizontal growth phase follows vertical growth and invasion. Mechanical stress frequently causes superficial erosions or ulceration.

Prognosis is dependent on the degree of development and invasion. Mechanical stress and usually delayed diagnosis are unfavorable factors.

5.Other malignant melanomas

These include melanoma of the retina and conjunctivae, as well as oral and genital mucosae.

A special mention must be also made of the amelanotic malignant melanoma, which raises great problems of diagnosis. It develops as pink or red nodule, usually on extremities. Its prognosis is worse than in other tumors, most probably due to its late recognition or misdiagnosis.

At last, in some cases, melanoma metastasis can be diagnosed without evidence of the primary tumor.

As melanoma is only curable in early stages, a correct clinical diagnosis is essential and should be made as early as possible. The differential diagnosis should be made in the first place with: 1) nevus cell naevi (dysplastic NCN, Spitz nevus, blue naevus); 2) pigmented epithelial and adnexal tumors (pigmented verruca seborrhoica, pigmented basalioma, irritated veruca vulgaris with hemorrhage, keratoacanthoma, pigmented spinalioma); 3) vascular lesions (thrombosed hemangioma, angiokeratoma, glomus tumor, kaposi sarcoma and subungual or subcorneal haematoma); 4) dermal tumors (pigmented dermatofibroma, pigmented hystiocytoma, neurofibroma).

1.3.2. Prognostic parameters

Malignant melanoma is a tumor which is curable with surgery alone with high survival rates when diagnosed early, and is highly resistant to therapy and in most cases fatal, when in advanced stages.

Melanoma can spread mostly lymphatically but also hematologically, and late metastases, developing years after the excision of primary tumor have been described. Metastasing occurs mostly to (in order of frequency) skin, lymph nodes, lungs, liver, bones and brain.

The main prognostic factors for primary malignant melanoma are the tumor thickness, assessed histologically (Breslow index) and tumor extension. According to these two parameters, the Commission for Malignant Melanoma of the German Society for Dermatology (DDG) has proposed a TNM staging classification of MM (Table 4):

Table 4. Clinical staging and survival rate in MM (ORFANOS, 2001)

Stage	*Primary tumor*	*Lymph nodes*	*Distant metastases*	*Survival rate at 10 years*
I a	pT1 (< 0.75 mm)	N0	M0	97 %
I b	pT2 (0.76-1.5 mm)	N0	M0	90 %
II a	pT3 (1.5-4 mm)	N0	M0	67 %
II b	pT4 (> 4 mm)	N0	M0	43 %
III a	in transit/satellite	N0	M0	28 %
III b	any	N1, N2	M0	19 %
IV	any	any	M1	3 %

Recently, a revised melanoma staging was approved by the American Joint Committee on Cancer (AJCC) and will become official in 2002. It incorporates as independent prognostic factors the tumor thickness (Breslow) and the presence of tumor ulceration, the number of metastatic lymph nodes and the delineation between clinically occult (microscopic) and clinically apparent (macroscopic) nodal metastasis, the site of distant metastases and the LDH serum level (BALCH *et al.,* 2001*;* KIM *et al.,* 2002*).*

Further parameters, proposed by different studies, such as Clark invasion level of tumor, localization of primary tumor (with assumed worse prognostic for the tumors in BANS region: (**b**ack, **a**rms, **n**eck, **s**calp), mitotic index and sex (with about 10 % higher survival rates for women) have a controversial prognostic relevance.

Detection of circulating tumor cells in patients' bloodstream by the mean of PCR analysis, is performed only in few centers and its prognostic value is still being evaluated (FARTHMANN *et al*., 1998).

1.3.3. Therapeutic procedures

As mentioned above, melanoma can be completely cured only in early stages, through total surgical removal. The early recognition of melanoma is therefore essential. Intensive educational programs for physicians as well as for the population, led to an improvement of the early diagnosis of melanoma, and consequently to the lowering of the mortality rate of melanoma over the last two decades, despite a continuously growing incidence of the tumor. Most of the new melanoma cases are currently being diagnosed in first and second stages.

As soon as the metastasing did occur however, the survival chances drop dramatically and the currently available therapeutic procedures (chemotherapy, immune therapy, radiation) have rather a palliative role.

1.3.3.1 Surgical therapy

The total tumor excision, with appropriate safety margin, in local anesthesia and with primary wound closure is the standard and most important therapeutic procedure in malignant melanoma. The safety margin is dictated by the estimated tumor thickness (Table 5) (ORFANOS, 2001).

When the diagnostic is not certain, a two-steps intervention can be accepted, with a first excision with narrow margin, followed, after histological confirmation of diagnosis, by a second excision, with appropriate safety margin. A second excision is also recommended when, by misdiagnosis, the primary excision of the tumor occurred with insufficient safety margin. This second step surgery should be performed within 4 weeks from the primary excision.

The surgical treatment can be completed by loco-regional radiation, in cases with difficult localization of the tumor, incomplete resection, or complex lymph drainage.

Table 5. Recommended safety margins by excision of primary MM

tumor thickness (mm) (Breslow)	safety margin (cm)
<1	1
1-4	2
>4	3

1.3.3.2 Elective lymph node dissection (ELND)

In contrast to the earlier approach, which consisted of systematic resection of the regional lymph nodes, the last years have imposed the sentinel lymph node biopsy (SLNB) as a more specific and minimally invasive method for detection of lymph node micrometastasis. This technique identifies scintigraphically the first draining lymph node of the tumor region, (the so-called sentinel node) which is then removed and histologically and immunohistologically investigated. Only if the presence of melanoma micrometastasis in the sentinel node is proven, the radical dissection of the remaining regional lymph nodes follows. This procedure avoids systematic mutilating surgery without proven clinical benefit.

The sentinel node detection should be performed optimally before excision of primary tumor, in order to avoid lymph drainage alteration through surgery. The SLNB is recommended for tumors thicker than 1 mm, but in some centers, it is performed already by 0.75 mm tumor thickness. Although the therapeutic benefit of ELND is still discussed, the SLN biopsy in contrast has become a major staging procedure, the nodal status being a powerful outcome predictor in MM patients.

1.3.3.3 Adjuvant therapy

The adjuvant therapy in MM is recommended after total removal of tumor, in tumor free patients with high risk tumor stages (II, III) and its scope is to reduce the risk of developing new metastases.

Various classes of therapeutic agents show a certain antitumor activity in MM. The most important are alkylating agents: chloroethylating agents (nitrosourea derivatives such as BCNU, CCNU, fotemustine and cystemustine) and methylating agents (DTIC, procarbazine and

temozolomide), vinca alkaloids (vindesine), taxanes (paclitaxel, docetaxel) and platinum derivatives (cisplatin). At the moment, there is no standard adjuvant therapy regimen with confirmed survival benefit.

The alkylating agents act through alkylation of DNA, with formation of methyl/ chlorethyl adducts in O_6 position of guanine. The methyl adducts induce an inefficient cycling of the mismatch repair pathway, which ultimately leads to DNA strand breaks, activating of poly-(ADP-ribose) polymerase and apoptotic cell death. The chlorethyl adducts, in change, induce formation of crosslinks between the twin DNA strands, which disrupt DNA synthesis and cause chromosomal aberration, rearrangements, and strand breaks, with cytotoxic effect.

The taxanes as well as the vinca alkaloids exert their cytotoxic effect by stabilizing polymerized microtubules during the cell mitosis, arresting thus the cell cycle in metaphase.

The platinum derivatives act also as alkylating agents.

In the current practice, different mono- as well as polychemotherapy regimens are used, with controversial results. The most frequently used single agent for adjuvant therapy is dacarbazine (DTIC). Polychemotherapy regimens include the BHD scheme (BCNU, hydroxyureea, DTIC), the BOLD scheme (bleomycin, vincristine, lomustine, DTIC), the DCTB scheme (Dartmouth regimen) (DTIC, BCNU, cisplatin and tamoxifen) and the DVP combination (DTIC, vindesine, cisplatin). There is a great variety in the chemotherapy or immuno-chemotherapy regimens used for the postoperative clinical management of MM in stages II and III. Although a benefit in prolonging the disease free interval was reported by different trials with all of these adjuvant therapy regimens, an improvement of overall survival rate remains controversial (BECKER *et al*., 2000).

A new development in the last decade was the introduction in the secondary prevention schemes of the α-interferon (α-IFN). The immunoregulatory and antiproliferative functions of IFN have been well documented *in vitro* and *in vivo* in melanoma, and its ability to stimulate the host's antitumoral immune response seems to play the central part in the activity of IFN in melanoma. IFNα is now used alone or in combination with the other classical chemotherapy regimens, defining the so-called biochemotherapy or immunochemotherapy. It is to date the only agent approved for adjuvant therapy in MM by the FDA. Its use is limited by its many side-effects which include flue like syndrome, headache, fever and asthenia, depression, hepatotoxicity and myelosuppression.

There is no consensus regarding the most effective dosage of IFN, and despite encouraging results from several clinical trials, the current data about real clinical benefit of IFN in melanoma patient are still contradictory and further investigation in appropriate large clinical trials is required.

1.3.3.4. Palliative therapy of metastasing melanoma (stage IV)

In the metastasing stage, the therapy of MM is basically palliative, and despite all efforts in finding new treatment strategies, the prognosis of these patients remains poor, with a reported median survival ranging from 6 to 9 months. In an early phase, a temporary remission and survival extension can be in some cases obtained. In late stages, the therapy concentrate on conserving an acceptable life's quality.

Surgical treatment

Surgical removal of regional or distant metastases is recommended, with influence on the prognosis, if only one organ is involved, the number and location of metastases allow complete resection, and no progress of the disease has been registered in the 3 months preceding the intervention.

Surgery can also be used with palliative intention, for preserving organ function or life's quality in terminal stages.

Radiation

Although melanoma shows and rather poor response to radiation, radiotherapy can be used for the stabilization of bone metastases, for delaying the progression of cerebral metastases, to complete the surgical lymphadenectomy or, palliative, to reduce pain and preserve organ function.

Chemotherapy

Chemotherapy is currently the main therapeutic approach of metastasing MM. The response rate to the various existing regimens is however confined to 20-35 % of cases, with complete remission in less than 50 % of the responders, so that the poor prognosis of late stage MM is hardly influenced.

In practice, both monochemotherapy as well as polychemotherapy regimens are used, with controversial if any difference in terms of survival so that the therapy choice is made mostly on individual basis.

As single agent therapy, DTIC is the most frequently used drug, with response rate of about 20 %. It can be administrated in one-day or in five-days schemas, without proven difference in outcome. Main adverse effects include nausea, photosensitivity, occasionally hepatotoxicity and rarely Budd-Chiari syndrome.

Other drugs used for monochemotherapy are BCNU, vindesine and fotemustine, with response rates ranging from 10 % to 20 %. The later is especially effective against intracerebral metastases. IFNα can be used in combination with any of these agents.

The polychemotherapy schemes are the same as those proposed for the adjuvant therapy. Less toxic regimens, with better tolerance, such as BHD can be used in earlier phases; by disease progression, more aggressive schemes, such as DVP, BLOD or Dartmouth, are preferred, being associated with more severe systemic adverse effects. The most frequent toxic effects, which limit the use of these drugs are myelosupression and hepatotoxicity, neurotoxicity, and nephrotoxicity, nausea, vomiting and hair loss.

Any of these polychemotherapy schemes can be administrated in association with αIFN, the efficacy of such immunochemotherapy combinations being still under clinical evaluation. A further promising approach, though burdened with high toxicity appears to be the combination of cisplatin, vinblastine, DTIC, with IFNα and Il2 in high doses, and is currently the subject of investigation (BECKER *et al*., 2000).

1.3.3.5 Experimental therapeutic approaches

The high mortality rate of the MM, the poor efficacy of chemotherapy in advanced malignant melanoma and the high toxicity of the classical regimens has stimulated a rich and intensive search for new therapeutic methods such as gene therapy or specific immunotherapy for fighting the spreading of the tumor. Most of these methods are still experimental.

Gene therapy has evolved following the development of new techniques of manipulation of gene expression such as gene transfection or mRNA antisense nucleotides technology. It is based on the attempt to fight the tumor by down- or up-regulating the expression of molecules

involved in tumorigenesis, growth and metastasing. The principal strategies of genetically targeting the tumor, which are currently investigated are:

1. *Correcting the genetic defect* responsible for the development of cancer cells. This can be achieved by interfering with the oncogene expression (such as Ras, Rho, c-Myc) or by restoring the expression of tumor suppressor genes (such as p53, or INK4a/p16).

2. *Molecular chemosensitization.* The principle of this strategy is to transduce cancer cells with genes encoding molecules which render cells sensitive to the proapoptotic effect of different agents. Such "suicide" molecules currently investigated are enzymes that can transform a non-toxic prodrug in a toxic agent (e.g. herpes virus thymidine kinase, active on ganciclovir), death receptors or death receptor ligands (Trail, CD95/FasL) or tumor suppressor genes (p53). Other *in vitro* and *in vivo* studies investigate the role of antisense method in blocking the overexpression of antiapoptotic molecules such as the Bcl-2 protein family, and thus sensitizing melanoma cells to drug induced apoptosis (SCHNEEBERGER *et al*., 2000*)*.

3. *Blocking the function of gene products associated with tumor progression.* These approaches attempt the regulation of expression of molecules involved in the neoangiogenesis, alteration in adhesion molecules pattern or activation of proteolytic enzymes, which are central processes in tumor invasion and metastasis. Among these, inhibition of tumoral angiogenesis seems the principal candidate for developing new anticancer therapies, with much attention focusing currently on interventions in the VEGF/VEGFR1/-R2 ligand-receptor system (FOLKMAN*,* 1995*;* SKOBE *et al,* 2001*)*.

Although promising in preclinical studies, the clinical use of these methods is seriously limited by the poor efficacy, stability and tumor- specificity of the current available gene transfer techniques.

The immunotherapeutic concept is based on the ability of the human immune system to detect and to respond to the presence of malignant cells. A very intensive experimental work is currently investigating the possibilities of stimulating, by means of antigenic cancer molecules, a tumor-specific cell-mediated immune response .

One main direction in the immunotherapy research is the development of cytokine based vaccines. These imply the injection of autologous melanoma cells genetically engineered to overexpress immunostimulatory molecules, such as IL2, IFNγ, TNFα, or M-CSF and GM-CSF.

A second approach consists in the identification of new tumor antigens, able to induce a tumor-specific immune response. The melanoma antigens so far identified include non-mutated melanocyte differentiation antigens (Pmel17/gp100, Mart-1/Melan-A, tyrosinase, TRP1 and 2),

cancer testis antigen families (MAGE, BAGE, GAGE) or products of mutated ubiquitously expressed genes (β-cathenin, cdk, FLICE). T cell immunity to these antigens can be elicited by injection of tumor antigen derived peptides, administration of viral vectors engineered to express a given antigen, or injection of a plasmid-type reporter gene construct containing the DNA sequence encoding the required antigen.

A further technique, which enjoys much attention in present, is the "vaccination" of patients with autologous dendritic cells, generated and maturated *ex vivo* and loaded with different combinations of tumor antigens (NESTLE *et al.,* 1998).

Although very successful in preclinical models, all these attempts raise great difficulties by translation into the clinic, mainly due to the lack of efficiency, stability and selectivity of the current available techniques of antigen delivery. Additionally, tumoral mechanisms of escaping the immune surveillance may also be responsible for the poor correlation between inducing an immune response and clinical improvement.

1.4 OBJECTIVE OF THE STUDY

Malignant melanoma is an aggressive tumor, with a high mortality rate in advanced stages. The current therapeutic strategies failed to show a definite influence in improving the survival rates and are associated with high systemic toxicity, so that elaboration of new therapeutic alternatives is necessary.

The bisphosphonates are compounds that have been for long in clinical use for the therapy of bone-resorptive diseases and especially of tumor induced osteolysis, with excellent tolerance. Recently they have been proved to have a direct antitumoral effect, *in vitro* and *in vivo* in different types of human tumors, so that they appear to be in present an interesting starting point in developing new anticancer therapy. Thus, they may also represent an alternative for the treatment of melanoma.

At the moment, there are no available data concerning a possible effect of bisphosphonates in malignant melanoma. Therefore the present study aims to investigate *in vitro* the effect of bisphosphonates on melanoma cell lines. Several experimental approaches should explore the influence of bisphosphonates on cell proliferation, cell cycle progression and apoptosis induction in melanoma cell lines. Moreover, in order to obtain new indices concerning the underlying mechanism of action of these compounds, different bisphosphonates with different antiresorbtive potency and different pharmacodynamic properties should be studied.

This research should attempt to bring new insights into the intimate mechanism of action of bisphosphonates and to provide the basis for the further investigation of the potential role of these compounds in anti-melanoma therapy.