ISSN 0025-7680

SPECIAL ARTICLE MEDICINA (Buenos Aires) 1997; 57 (Supl. I): 65-75

MECHANISMS OF ACTION OF THE BISPHOSPHONATES

HERBERT FLEISCH

Department of Pathophysiology, University of Berne, Berne, Switzerland

Abstract: Geminal bisphosphonates, usually called bisphosphonates, are synthetic compounds characterized by a P-C-P bond. Many such bisphosphonates have been synthesized, each of them having its own physicochemical and biologic characteristics. This implies that it is not possible to extrapolate from the results of one compound to others with respect to their actions. Bisphosphonates can exert physicochemical effects very similar to those of polyphosphates, binding to the surface of calcium phosphate crystals and inhibiting their formation and aggregation as well as their dissolution. Many of the bisphosphonates are very powerful inhibitors of bone resorption. This is seen both in normal animals and in animals where bone resorption is simulated by various means. Thus, they are active in various models of human diseases, such as hyperparathyroidism, tumoral bone disease and osteoporosis. They not only prevent bone loss, but actively increase bone mass and improve the biomechanical properties of the skeleton. The activity varies greatly from compound to compound, the newest bisphosphonates being 5,000 to 10,000 times more active than etidronate, the first bisphosphonate described. The mechanism of action appears to be complex. It involves: a) a direct effect on the osteoclast activity. b) A direct and indirect effect on the osteoclast recruitment. The latter is mediated by cells of the osteoblastic lineage and involves their production of an inhibitor of osteoclastic recruitment. c) A shortening of osteoclast survival by apoptosis. Large amounts of bisphosphonates can also inhibit mineralization through a physicochemical inhibition of crystal growth. Bisphosphonates are used therapeutically in humans to decrease bone resorption, among others in Paget's disease, tumor bone disease, and recently osteoporosis. Etidronate is also sometimes used to prevent ectopic calcification.

Key words: bisphosphonates, mechanism of action

The bisphosphonates are a new class of drugs which have been developed for use in various diseases of bone, tooth, and calcium metabolism. This article will cover the mechanisms of action of these compounds. For more information on both the preclinical and clinical aspects, the reader is referred to some recent reviews[15,16,25,47].

Our knowledge of the biologic characteristics of bisphosphonates dates back to the last three decades, the first report[17] having appeared in 1968. The concept has been derived from our earlier studies on inorganic pyrophosphate. We had found that this compound prevents both the formation and dissolution of calcium phosphate in vitro. Since we found pyrophosphate to be present in biological fluids, we suggested that it might be a physiologic regulator of calcification and perhaps also of decalcification in vivo[20]. Due to its rapid hydrolysis, pyrophosphate found therapeutic use only in scintigraphy and against dental calculus (it is now used worldwide as an anti-tartar agent in toothpastes).

This prompted the search for analogs which would display similar physicochemical activity but resist enzymatic hydrolysis and, therefore, would not be broken down metabolically. The clue came from the chemical industry. Thus pyrophosphate and polyphosphates were used for a long time for

Postal addres: Dr. Herbert Fleisch, Departament of Pathophysiology, University of Berne, Murtenstrasse 35, CH-3010, Berne, Switzerland

various industrial purposes, among others to prevent the precipitation of calcium carbonate in washing powders, water, and oil brines. Bisphosphonates were found to have similar effects and were thus good candidates for fulfilling the above mentioned conditions in biology. These compounds have been known to the chemical industry for a long time, since the first bisphosphonate was synthesized in Germany in 1865[39], etidronate being first synthesized in 1897[62].

Chemistry and physicochemical effects

Bisphosphonates, erroneously called diphosphonates in the past, are compounds characterized by two C-P bonds. If the two bonds are located on the same carbon atom, the compounds are called geminal bisphosphonates and are analogs to pyrophosphate, which contains an oxygen instead of a carbon atom (Fig 1). Only geminal bisphosphonates seem to have a strong activity on the skeleton.

The P-C-P structure allows a great number of possible variations, especially by changing the two lateral chains on the carbon. The following bisphosphonates have been investigated in humans in bone disease. Six of them are now commercially available (Fig. 2).

Each bisphosphonate has its own physicochemical and biologic characteristics, which implies that it is not possible to extrapolate from the results of one compound to others with respect to its actions. However some characteristics apply to most or even all bisphosphonates. The P-C-P bond is stable to heat and most chemical reagents and is completely resistant to enzymatic hydrolysis. The bisphosphonates have in general a strong affinity for metal ions such as calcium and iron. Some uncertainty still exists as to their state when in solution. Indeed, they are only partially ultrafiltrable in aqueous solutions as well as in plasma[63], partly because of the formation of complexes and aggregates, partly because of binding to proteins, especially albumin[36].

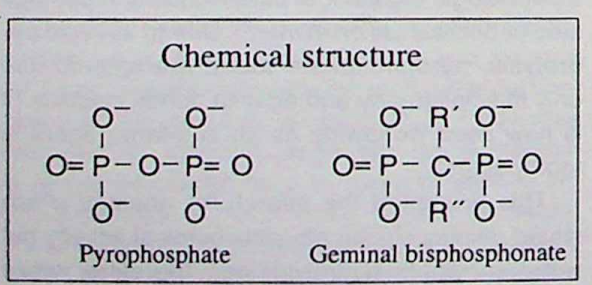


Fig. 1. Chemical structure of pyrophosphate and bisphosphonates.

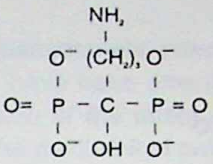
As was anticipated, the physicochemical effects of most of the bisphosphonates are very similar to those of pyrophosphate. Thus, most of them inhibit the formation and aggregation of calcium phosphate crystals[18,21,23] and slow down their dissolution[19,48]. All these effects are related to the marked affinity of these compounds for the surface of solid phase calcium phosphate[33] where they act as a crystal poison on both growth and dissolution.

Biological effects

Inhibition of bone resorption. Bisphosphonates proved to be very powerful inhibitors of bone resorption when tested in a variety of conditions, both in vitro and in vivo.

Tissue and organ culture. Bisphosphonates block bone resorption induced by various means in organ culture[19,46]. Unlike previous results, it was recently shown that, when using nine compounds varying in their activity by 5-6 orders of magnitude, there is a satisfactory correlation between the results obtained in vitro using the mouse calvaria system, and those found in vivo[27]. An inhibition is also found when the effect of isolated osteoclasts is investigated on various mineralized matrices in vitro[13,49,50]. However, only certain of the models used give a relation of potency with that found in vivo.

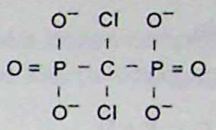
Normal animals. In normal growing rats, the bisphosphonates block the degradation of both bone and cartilage, thus arresting the remodeling of the metaphysis, which becomes club-shaped and radiologically denser than normal[53]. This effect is used as a model to study the potency of new compounds[52]. The inhibition of endogenous bone resorption has also been documented by ^{45}Ca kinetic studies[14,24], and by markers of bone resorption. The effect occurs within 24-48 hours and is therefore slower than that of calcitonin.



(4 -Amino - 1 - hidroxybutylidene)-bis-phosphonate

alendronate*

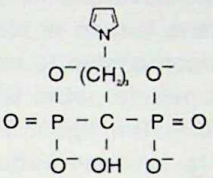
Gentili; Merck Sharp & Dohme



(Dichloromethylene)-bis-phosphonate

clodronate*

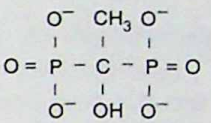
Astra; Boehringer Mannheim; Gentili; Leiras; Rhône-Poulenc Rorer



[1- Hidroxy-3-(1-pyrrolidinyl)-propylidene] bis-phosphonate

EB-1053

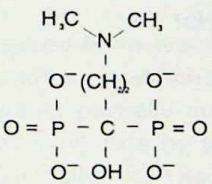
Leo



(1-Hydroxyethylidene)-bis-phosphonate

etidronate*

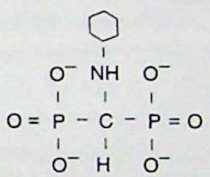
Gentili; Procter & Gamble



[3- (Dimethylamino)-1-hydroxy-propylidene] bis-phosphonate

olpadronate

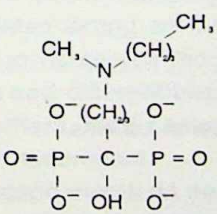
Gador



[(Cycloheptylamino)-methylene] bis-phosphonate

incadronate

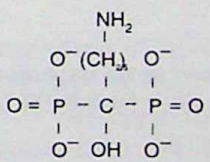
Yamanouchi



[1- Hidroxy-3-(methylpentylamino)-propylidene] bis-phosphonate

ibandronate*

Boehringer Mannheim



[6-Amino -1-hydroxyhexy-lidene] bis-phosphonate

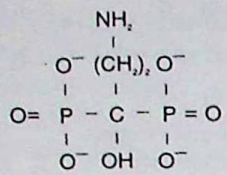
neridronate

Gentili

Fig. 2 a. Chemical structure of the bisphosphonates investigated for their effects in humans.
* Commercially available. (From Fleisch 1997).

The decrease in resorption is accompanied by an increase in calcium balance[24], and mineral content of bone. This is possible because of an increase in intestinal absorption of calcium, consequent on an elevation of $1,25(OH)_2$ vitamin D. In most cases this increase is, however, smaller than predicted, considering the dramatic decrease in bone resorption. The reason is that bone formation also decreases after a certain time.

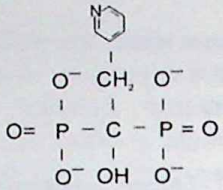
Of clinical importance is the recent finding that treatments which increase bone formation, such as prostaglandins, IGF-1, and PTH, are still effec-



(3-Amino - 1 - hidroxypropy-lidene) bis-phosphonate

pamidronate*

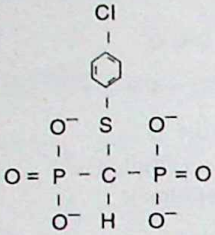
Ciba - Geigy; Gador



(1- Hidroxy-2-(3-pyridinyl)-ethylidene) bis-phosphonate

risedronate

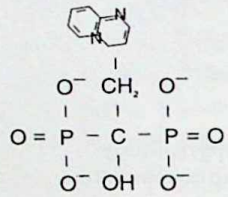
Procter & Gamble



[[(4-Chlorphenyl)thio]-methylene] bis-phosphonate

tiludronate*

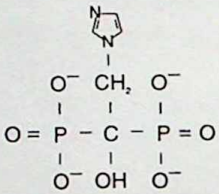
Sanofi



[1- Hidroxy-2-imidazo-(1,2-a) pyridin-3-ylethylidene] bis-phosphonate

YH 529

Yamanouchi - Hoechst



[1- Hidroxy-2(1H-imidazol-1-y) ethylidene] bis-phosphonat

zoledronate

Giba - Geigy

Fig. 2 b. Chemical structure of the bisphosphonates investigated for their effects in humans.
* Commercially available (from Fleisch 1997).

tive in rats treated with bisphosphonates, resulting in an additive effect of the two treatments on bone mass.

Animals with experimentally increased bone resorption. Bisphosphonates can also prevent experimentally induced increases in bone resorption. Thus they impair, among others, resorption induced by agents such as parathyroid hormone (19,48), 1,25$(OH)_2$ vitamin D, and retinoids[59]. The effect on retinoid-induced hypercalcaemia has been used to develop a powerful and rapid screening assay for new compounds[59]. Of special clinical interest is the effect in models of osteoporosis and tumor bone disease.

Osteoporosis. The bisphosphonates are effective in preventing bone loss in a number of experimental osteoporosis models. These include: sciatic nerve section, which was the first model investigated[42], spinal cord section, hypokinesis, ovariectomy[3,64], heparin, lactation, and corticosteroids[32]. The effect on bone loss induced by a low Ca diet is ambiguous. All bisphosphonates investigated, such as, in alphabetical order, alendronate, clodronate, etidronate, ibandronate, incadronate,

olpadronate, pamidronate, risedronate, tiludronate, and YH 529, have been effective.

The question of the effect of the bisphosphonates upon the mechanical properties of the skeleton has been addressed only recently. This issue is of importance since long-lasting, strong inhibition of bone resorption can lead to increased fragility both in animals and in humans. It is now clear that, when not given in excess, bisphosphonates induce an improvement of biomechanical properties both in normal animals and in experimental models of osteoporosis. This is the case, in alphabetical order, of alendronate, clodronate, etidronate (although with this drug the effect is more ambiguous because at higher doses it is obscured by an inhibition of mineralization), incadronate, neridronate, olpadronate, pamidronate, tiludronate, and YH 529. It is seen in various animals such as the rat, the chick and the baboon[1,3,12,40,57].

Tumor-induced bone resorption . Bisphosphonates also inhibit tumoral bone resorption. Various bisphosphonates partially correct the hypercalcaemia induced in rats by subcutaneously implanted tumor cells[34,37]. The effect is generally more pronounced in calciuria than in calcemia. This is explained by the fact that hypercalcaemia is often due to the systemic production of PTH-related peptide which increases both bone resorption and tubular reabsorption of calcium, the bisphosphonates acting on the former. This has been shown in numerous models using different tumoral cells and different bisphosphonates such as, among others, clodronate, etidronate, pamidronate, and risedronate.

The bisphosphonates do not directly inhibit the multiplication of tumor cells and are therefore not active on the tumor itself, but exert their action by inhibiting the osteolytic process. However, multiplication of tumor cells may be decreased as a secondary consequence, possibly in part because of a decrease in the number of local cytokines which stimulate tumor cell replication and are released when bone is resorbed.

Potency of bisphosphonates. With the increase in the clinical interest for bisphosphonates, a great effort has been devoted to synthesizing new compounds with a greater potency for decreasing bone resorption. This has been successful and up to now, over a dozen compounds have been tested in humans with potencies ranging from 1 to 10,000 (Fig. 3).

Mechanisms of action

The mechanisms of action of the bisphosphonates in bone resorption is not yet completely elucidated. Effects can be distinguished at the tissue, cell, and molecular levels.

Tissue. As described above, bisphosphonates decrease bone resorption, leading to a decrease in both bone modeling and remodeling. The latter consists of the destruction and reconstruction of a certain quantity of bone in a specified location. The morphological dynamic structure of this quantity of bone is the «basic multicellular unit» (BMU). This process of remodeling, which occurs both in cortical and trabecular bone, begins by bone being eroded by osteoclasts. In a second step, the defect thus created is refilled by osteoblasts. This is the basis for the so-called «coupling» between bone resorption and bone formation. Bisphosphonates decrease the number of active BMUs as well as the activity of the osteoclasts in them. It is thus understandable why the bisphosphonates also decrease bone formation[26]. This is, however, a secondary event due to the decrease in the number of BMUs, and not a direct effect on the process of bone formation per se. Thus the main effect of the bisphosphonates is to reduce bone turnover.

In osteoporosis there is a negative bone balance at the level of each BMU, less bone being formed than destroyed. One of the mechanisms of bisphosphonates that prevents bone loss is explained by the decrease in turnover. In addition, the bisphosphonates also act at the individual BMU by decreasing the depth of the resorption site[3,5,56]. Both effects will lead to a decrease in

Potency to inhibit bone resorption					
-1x	-10x	- 100 x	> 100- < 1000 X	> 1000- < 10 000 X	> 10 000-
Etidronare	Clodronate Tiludronate	Pamidronate Neridronate	Alendronate EB-1053 Incadronate Olpadronate	Ibandronate Risedronate	YH 529 Zoledronate

Fig. 3. Potency of the major bisphosphonates to inhibit bone resorption in the rat (From Fleisch (1997).

bone loss and a smaller number of trabecular perforations, thus inhibiting the decrease in bone strength and the occurrence of fractures.

The actual increase in bone mass, seen both in animals and humans, has probably various causes. One explanation is that the decrease in bone resorption is not immediately followed by the «coupling-induced» diminution of formation, bringing a temporary gain in calcium balance through the reduction of the so-called remodeling space. Another possibility is that a lower turnover will lengthen the life span of the BMU, thus permitting it to mineralize more completely. The third explanation is that by reducing resorption less than formation at the individual BMU, bone balance is thus increased (Figure 4). Actually the amount of bone formed at the level of the BMU, assessed by the thickness of the packet of bone formed, is not decreased[3,5,56], although total bone formation is decreased[3,5,24,56]. If anything it seems to be increased[3,5,56], but this effect is small and needs to be confirmed.

Cell. There is a general consensus that the bisphosphonates act through the osteoclasts. This could occur in the following ways: a) inhibition of osteoclast recruitment to the bone surface; b) inhibition of osteoclast activity on the bone surface; c) shortening of the life span of the osteoclast; and d) inhibition of the mineral dissolution by alteration of its surface.

a) Osteoclastic recruitment has been found to be decreased in bone marrow culture[30], as well as in calvaria and metatarsal culture[4]. While some of this effect may be a direct one on osteoclast precursors, recent results show that when rat osteoblastic cells are briefly[49] or continuously[43] exposed to low concentrations of potent bisphosphonates, their conditioned medium contains a factor(s) that reduces osteoclastic bone resorption in culture. The decrease in resorption was subsequently found to be due to an inhibitor of osteoclast recruitment or survival, with a molecular weight between 1 and 10 kD, released by the osteoblasts[48,61]. Until now it is not known which cells of the osteoblastic lineage are involved in this mechanism (see Fig. 5).

b) An inhibition of osteoclastic activity has been shown in various models. The bisphosphonates alter the morphology of osteoclasts both in vitro[41,50] and in vivo[53]. The changes affect the number of osteoclastic nuclei[53], the cytoskeleton, especially actin[41] and the ruffled border which tends to disappear[41,45,50]. Furthermore, adding bisphosphonates to osteoclasts exposed in vitro to mineralized substrata like bone, dentine, or ivory leads to an inhibition of their resorbing activity[13,49,50]. This activity is still present when these substrata are exposed to the bisphosphonates before the osteoclasts are added, suggesting that the osteoclasts are inhibited when they come in contact with mineral containing bisphosphonate. Thus it was hypothesized that the bisphosphonates are

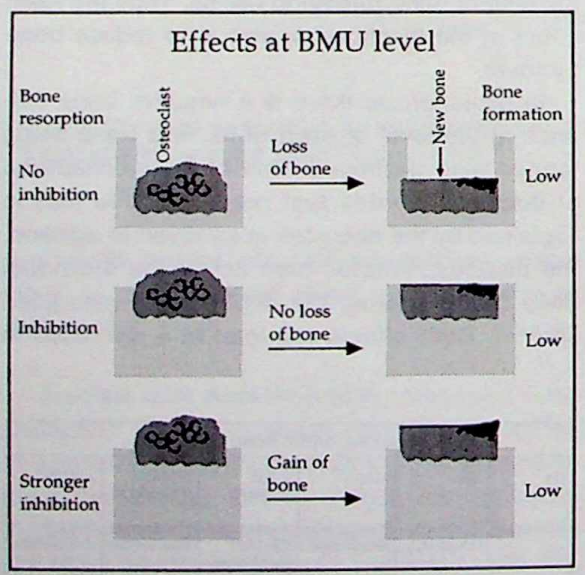


Fig. 4. Possible effect of bisphosphonates at the level of the individual BMU. (From Fleisch (1997).

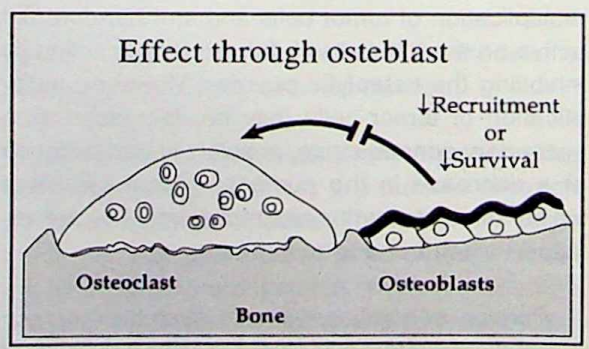


Fig. 5. Indirect effect of the bisphosphonates on the osteoclasts mediated by the osteoblasts. (From Fleisch (1997).

deposited into bone because of their strong affinity for the mineral, and that the osteoclasts are then inhibited when they engulf bone containing bisphosphonate (Fig. 6). This hypothesis is further supported by the recent finding that bisphosphonates, when administered in low amounts, preferentially deposit not in newly formed bone as previously thought, but under the osteoclasts[2,51]. The relative deposition in bone forming and bone resorbing areas depends upon the amount of bisphosphonate injected. The resorbing surface is preferentially labeled by radioactive bisphosphonate when the amount administered is small, as in the case of the more powerful bisphosphonates such as alendronate. The areas are about equal when large amounts are given, as in the case of etidronate[38]. It has been calculated that the concentration under the osteoclasts can reach very high values, in the range of 0.1-1 mM[51].

Shortening of the osteoclast life span. Recently it was shown that bisphosphonates increase osteoclastic apoptosis, thereby reducing their life span[31].

Alteration of bone mineral. Initially it was hypothesized that the bisphos-phonates would act by decreasing the rate of bone mineral dissolution. This mechanism appears to be of little or even no relevance. Thus there was no relation between the decrease of mineral dissolution in vitro and the inhibition of bone resorption in vivo when various bisphosphonates were investigated[55].

At present it is not known which of the various mechanisms, the direct effect on the osteoclast activity, the indirect effect on the osteoclast recruitment, or the effect on apoptosis is the most relevant in vivo.

Molecular level. Recently some progress has been made in the understanding of the chain of events leading to osteoclast inactivation, decreased formation and increased destruction. Many results point to the existence of some sort of «receptor» either at the cell surface or within the cell. Since bisphosphonates are taken up by cells[10,11,38,51], some effect within the cell is a possibility. Indeed, the sodium-independent extrusion of acid by osteoclasts is inhibited by alendronate[65]. Furthermore, tiludronate inhibits the vacuolar type proton ATPase of osteoclasts[8]. Lastly, bisphosphonates inhibit protein tyrosine phosphatases[54] which appear to be involved in osteoclast activity. The various cellular mechanisms are depicted in Fig. 7.

Effect on bone formation

Until recently it was thought that bisphosphonates did not affect bone formation. However, the results on the BMU suggest that possibly some stimulating effect might be present. It might be relevant in this regard to mention that bisphosphonates have been shown to increase the proliferation of cartilage cells[9], as well as the biosynthesis of collagen and proteoglycans by bone and cartilage cells[28,29] in vitro. Furthermore, alendronate can increase colony formation of osteoblasts (60) and the formation of mineralized

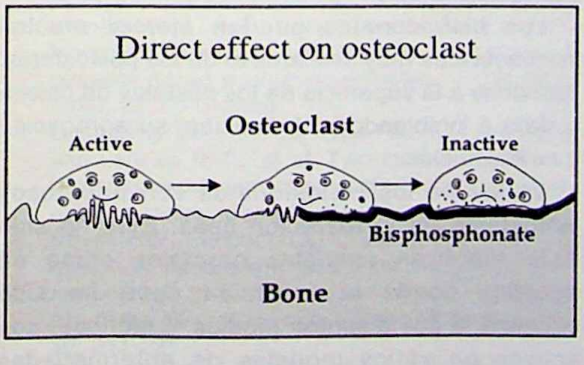


Fig. 6. Direct effect of bisphosphonates on the osteoclasts. (From Fleisch (1997).

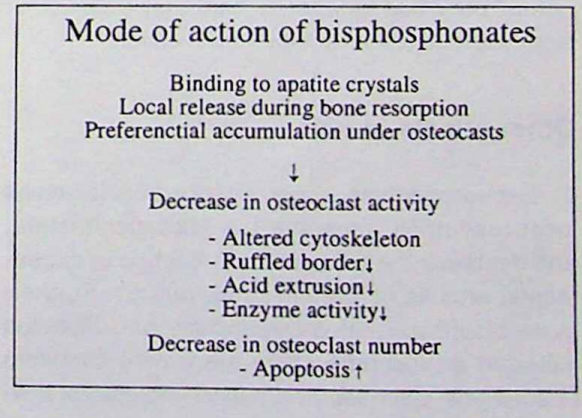


Fig. 7. Possible biochemical action of bisphosphonates on the osteoclast. (From Fleisch (1997).

nodules in human cell cultures in vitro, perhaps due to an increased formation of FGFβ[26]. Thus there is a possibility that, under certain circumstances, bisphosphonates also act by increasing bone formation.

Inhibition of calcification

Like pyrophosphate, bisphosphonates very efficiently inhibit calcification in vivo. Thus, among others, they prevent experimentally induced calcification of many soft tissues, both when given parenterally or orally[18], as well as ectopic ossification. Topical administration leads to a decreased formation of dental calculus[6].

If administered in sufficient doses, bisphosphonates can also impair the mineralization of normal calcified tissues such as bone, cartilage, dentine, and enamel[35,53]. The quantity required to produce this effect varies according to the bisphosphonate used, the animal species, and the length of treatment. In contrast to bone resorption where the different compounds vary greatly in their activity, they do not vary in the case of inhibition of mineralization. For most species the effective daily dose is in the order of 5-20 mg of compound phosphorus per kg parenterally. There is a close relationship between the ability of an individual bisphosphonate to inhibit calcium phosphate in vitro and its effectiveness on calcification in vivo[58]. Therefore, the mechanism is likely to be physicochemical. The inhibition of mineralization can lead to fractures and to their impaired healing. Although the inhibition of mineralization is eventually reversed after discontinuation of the drug, the propensity to inhibit the calcification of normal bone has hampered the therapeutic use of bisphosphonates in ectopic calcification.

Other effects in vivo

Bisphosphonates, especially risedronate, inhibit local resorption, preserve the joint architecture, and decrease the inflammatory reaction in experimental arthritis by Freund's adjuvant[22,44]. Furthermore, bisphosphonates and phosphono-sulfonates linked to an isoprene chain are potent inhibitors of squalene synthase and hence cholesterol lowering agents in the animal[7].

Conclusion

The bisphosphonates are very powerful inhibitors of bone resorption and can, at high doses, also inhibit mineralization. Since the dose which inhibits mineralization is similar for all bisphosphonates, while that inhibiting bone resorption varies from 1 to 10,000, no impairment of normal calcification is present with the newer, more powerful compounds.

The mechanisms of action are still not completely understood. While they are purely physicochemical, namely an inhibition of crystal growth for mineralization, they are cellular for bone resorption. These cellular effects include a decrease in osteoclast recruitment, partially through an osteoblast-mediated mechanism, an increased apoptosis, and a decrease in osteoclastic activity.

In view of their action, the bisphosphonates have been developed into a group of very efficient drugs for the treatment of diseases characterized by increased bone resorption, such as Paget's disease, tumor bone disease and, more recently, osteoporosis.

Resumen

Mecanismo de acción de los bisfosfonatos

Los bisfosfonatos geminales, denominados simplemente bisfosfonatos, son compuestos sintéticos caracterizados por la ligadura P-C-P. Han sido sintetizados varios de tales bisfosfonatos, cada uno de ellos presentando sus propias características fisicoquímicas y biológicas. Esto significa que con respecto a sus acciones no es posible extrapolar los resultados de un compuesto a otro.

Los bisfosfonatos pueden ejercer efectos fisicoquímicos muy similares al de los polifosfatos, ligándose a la superficie de los cristales de calciofosfato e inhibiendo su formación, su agregación y su disolución.

Varios de los bisfosfonatos son poderosos inhibidores de la resorción ósea. Esto ha sido visto tanto en animales normales como en aquellos donde la resorción ósea ha sido estimulada por distintos medios. Entonces, son activos en varios modelos de enfermedades humanas tales como hiperparatiroidismo, tumores

óseos y osteoporosis. Los bisfosfonatos no sólo previenen la pérdida de masa ósea, sino que activamente aumentan la masa ósea y mejoran las propiedades biomecánicas del esqueleto. La actividad difiere considerablemente de compuesto en compuesto, siendo los compuestos más novedosos de 5.000 a 10.000 veces más activos que el etidronato, el primer bisfosfonato descrito.

El mecanismo de acción parece ser complejo. Abarca: a) El efecto directo sobre la actividad de los osteoclastos. b) El efecto directo e indirecto sobre el reclutamiento de los osteoclastos, siendo el último mediado por células del linaje osteoblástico y comprende la producción de un inhibidor del reclutamiento osteoclástico. c) El acortamiento de la supervivencia de los osteoclastos por apoptosis.

Las grandes cantidades de bisfosfonatos pueden también inhibir la mineralización por una inhibición fisicoquímica del crecimiento del cristal.

Los bisfosfonatos son utilizados en la terapéutica humana para disminuir la resorción ósea, entre otras en la enfermedad ósea de Paget, en la patología tumoral y recientemente en la osteoporosis. El etidronato es también utilizado en ocasiones para prevenir las calcificaciones ectópicas.

References

1. Ammann P, Rizzoli R, Caverzasio J, et al. Effects of the bisphosphonate tiludronate on bone resorption, calcium balance, and bone mineral density. *J Bone Miner Res* 1993; 8: 1491-1498.
2. Azuma Y, Sato H, Oue Y, et al. Alendronate distributed on bone surfaces inhibits osteoclastic bone resorption in vitro and in experimental hypercalcemic models. *Bone* 1995; 16: 235-245.
3. Balena R, Toolan BC, Shea M, et al. The effects of 2-year treatment with the aminobisphosphonate alendronate on bone metabolism, bone histomorphometry, and bone strength in ovariectomized nonhuman primates. *J Clin Invest* 1993; 92: 2577-2586.
4. Bonekamp PM, van der Wee-Pals LJA, van Wijk-van Lennep MML, et al. Two modes of action of bisphosphonates on osteoclastic resorption of mineralized matrix. *Bone Miner* 1986; 1: 27-39.
5. Boyce RW, Paddock CL, Gleason JR, et al. The effects of risedronate on canine cancellous bone remodeling: three-dimensional kinetic reconstruction of the remodeling site. *J Bone Miner Res* 1995; 10: 211-221.
6. Briner WW, Francis MD, Widder, JS. The control of dental calculus in experimental animals. *Int Dent J* 1971; 21: 61-73.
7. Ciosek CP Jr., Magnin DR, Harrity TW, et al. Lipophilic 1,1-bisphosphonates are potent squalene synthase inhibitors and orally active cholesterol lowering agents in vivo. *J Biol Chem* 1993; 268: 24832-24837.
8. David P, Nguyen H, Barbier H, Baron R. Tiludronate is a potent and specific inhibitor of the osteoclast vacuolar H⁺ATPase. *Bone (Tarrytown)* 1995; 16: 166S.
9. Evêquoz V, Trechsel U, Fleisch, H. Effect of bisphosphonates on production of interleukin 1-like activity by macrophages and its effect on rabbit chondrocytes. *Bone* 1985; 6: 439-444.
10. Fast DK, Felix R, Dowse C. The effects of diphosphonates on the growth and glycolysis of connective-tissue cells in culture. *Biochem J* 1978; 172: 97-107.
11. Felix R, Guenther HL, Fleisch, H. The subcellular distribution of [^{14}C]dichloromethylenebisphospho-nate and [^{14}C]1-hydroxyethylidene-1,1-bisphospo-nate in cultured calvaria cells. *Calcif Tissue Int* 1984; 36: 108-113.
12. Ferretti JL, Delgado CJ, Capozza RF, et al. Protective effects of disodium etidronate and pamidronate against the biochemical repercussion of betamethasone-induced osteopenia in growing rat femurs. *Bone Miner* 1993; 20: 265-276.
13. Flanagan AM, Chambers TJ. Dichloromethylenebisphosphonate (Cl_2MBP) inhibits bone resor-ption through injury to osteoclasts that resorb Cl_2MBP-coated bone. *Bone Miner* 1989; 6: 33-43.
14. Fleisch H. The bisphosphonate ibandronate, given daily as well as discontinuously, decreases bone resorption and increases calcium retention as assessed by ^{45}Ca kinetics in the intact rat. *Osteopor Int* 1996; 6: 166-170.
15. Fleisch H. Bisphosphonates: Mechanisms of action and clinical use. In: Principles of Bone Biology. JP Bilezikian, LG Raisz, GA Rodan (eds.) Academic Press San Diego London, 1996: pp. 1037-1052.
16. Fleisch, H. Bisphosphonates in bone disease. From the laboratory to the patient. Third Edition, The Parthenon Publishing Group Ltd., Carnforth, New York; 1997.
17. Fleisch H, Russell RGG, Bisaz S, et al. The influence of pyrophosphate analogues (diphosphonates) on the precipitation and dissolution of calcium phosphate in vitro and in vivo. *Calcif Tiss Res* 1968; 2(Suppl.): 10-10a.
18. Fleisch H, Russell RGG, Bisaz S, et al. The inhibitory effect of phosphonates on the formation of calcium phosphate crystals in vitro and on aortic and kidney calcification in vivo. *Eur J Clin Invest* 1970; 1: 12-18.
19. Fleisch H, Russell RGG, Francis M.D. Diphosphonates inhibit hydroxyapatite dissolution in vitro and bone resorption in tissue culture and in vivo. *Science* 1969; 165: 1262-1264.
20. Fleisch H, Russell RGG, Straumann F. Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. *Nature* 1966; 212: 901-903.
21. Francis MD. The inhibition of calcium hydroxyapa-tite

crystal growth by polyphosphonates and polyphosphates. *Calcif Tissue Res* 1969; 3: 151-162.
22. Francis MD, Hovancik K, Boyce RW. NE-58095: A diphosphonate which prevents bone erosion and preserves joint architecture in experimental arthritis. *Int J Tissue React* 1989; 11: 239-252.
23. Francis MD, Russell RGG, Fleisch H. Diphosphonates inhibit formation of calcium phosphate crystals in vitro and pathological calcification in vivo. *Science* 1969; 165: 1264-1266.
24. Gasser AB, Morgan DB, Fleisch HA, Richelle, L.J. The influence of two diphosphonates on calcium metabolism in the rat. *Clin Sci* 1972; 43: 31-45.
25. Geddes AD, D'Souza SM, Ebetino FH, Ibbotson KJ. Bisphosphonates: structure-activity relationships and therapeutic implications. In: Heersche JNM, Kanis JA (eds.) Bone and mineral research. Elsevier Amsterdam 1994; pp. 265-306.
26. Giuliani N, Girasole G, Pedrazzoni M, et al. Alendronate stimulates β-FGF production and mineralized nodule formation in human osteoblastic cells and osteoblastogenesis in human bone marrow cultures. *J Bone Miner Res* 1995; 10(Suppl. 1): S171, Abstract 129.
27. Green JR, Müller K, Jaeggi KA. Preclinical pharmacology of CGP 42,446, a new, potent, heterocyclic bisphosphonate compound. *J Bone Miner Res* 1994; 9: 745-751.
28. Guenther HL, Guenther HE, Fleisch H. The effects of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on collagen synthesis by rabbit articular chondrocytes and rat bone cells. *Biochem J* 1981; 196: 293-301.
29. Guenther HL, Guenther HE, Fleisch H. The influence of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on lysine hydroxylation and cross-link formation in rat bone, cartilage and skin collagen. *Biochem J* 1981; 196: 303-310.
30. Hughes DE, MacDonald BR, Russell RGG, Gowen M. Inhibition of osteoclast-like cell formation by bisphosphonates in long-term cultures of human bone marrow. *J Clin Invest* 1989; 83: 1930-1935.
31. Hughes DE, Wright KR, Uy HL, et al. Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo. *J Bone Miner Res* 1995; 10: 1478-1487.
32. Jee WSS, Black HE, Gotcher JE. Effect of dichloromethane diphosphonate on cortisol-induced bone loss in young adult rabbits. *Clin Orthop* 1981; 156: 39-51.
33. Jung A, Bisaz S, Fleisch H. The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals. *Calcif Tiss Res* 1973; 11: 269-280.
34. Jung A, Bornand J, Mermillod B, Edouard C, Meunier PJ. Inhibition by diphosphonates of bone resorption induced by the Walker tumor of the rat. *Cancer Res* 1984; 44: 3007-3011.
35. King WR, Francis MD, Michael WR. Effect of disodium ethane-1-hydroxy-1,1-diphosphonate on bone formation. *Clin Orthop* 1971; 78: 251-270.
36. Lin JH, Chen I-W, DeLuna FA. Nonlinear kinetics of alendronate. Plasma protein binding and bone uptake. *Drug Metab Dispos* 1994; 22: 400-405.
37. Martodam RR, Thornton KS, Sica DA, D'Souza SM, Flora L, Mundy GR. The effects of dichloromethylene diphosphonate on hypercalcemia and other parameters of the humoral hypercalcemia of malignancy in the rat Leydig cell tumor. *Calcif Tissue Int* 1983; 35: 512-519.
38. Masarachia P, Weinreb M, Rodan GA. Comparison of the distribution of ^{3}H-alendronate and ^{3}H-etidronate in rat and mouse bones. *Bone* 1996; 19: 281-290.
39. Menschutkin N. Ueber die Einwirkung des Chloracetyls auf phosphorige Säure. *Ann Chem Pharm* 1865; 133: 317-320.
40. Motoie H, Nakamura T, O'uchi N. et al. Effects of the bisphosphonate YM175 on bone mineral density, strength, structure, and turnover in ovariectomized beagles on concomitant dietary calcium restriction. *J Bone Miner Res* 1995; 10: 910-920.
41. Murakami HN, Takahashi T, Sasaki N. et al. A possible mechanism of the specific action of bisphosphonates on osteoclasts: tiludronate preferentially affects polarized osteoclasts having ruffled borders. *Bone (Tarrytown)* 1995; 17: 137-144.
42. Mühlbauer RC, Bauss F, Schenk R.et al. BM 21.0955, a potent new bisphosphonate to inhibit bone resorption. *J Bone Miner Res* 1991; 6: 1003-1011.
43. Nishikawa M, Akatsu T, Katayama Y. et al. Bisphosphonates act on osteoblastic cells and inhibit osteoclast formation in mouse marrow cultures. 1996; 18: 9-14.
44. Österman T, Kippo K, Laurén L, Hannuniemi R, Sellman R. Effect of clodronate on established adjuvant arthritis. *Rheumatol Int* 1994; 14: 139-147.
45. Plasmans CMT, Jap PHK, Kuypers W., Slooff TJJ. Influence of a diphosphonate on the cellular aspect of young bone tissue. *Calcif Tissue Int* 1980; 32: 247-256.
46. Reynolds JJ, Minkin C, Morgan DB, Spycher D, Fleisch H. The effect of two diphosphonates on the resorption of mouse calvaria in vitro. *Calcif Tiss* Res 1972; 10: 302-313.
47. Rodan GA, Fleisch HA. Bisphosphonates: mechanisms of action. *J Clin Invest* 1996; 97: 2692-2696.
48. Russell RGG, Mühlbauer RC, Bisaz S, William, DA, Fleisch H. The influence of pyrophosphate, condensed phosphates, phosphonates and other phosphate compounds on the dissolution of hydroxyapatite in vitro and on bone resorption induced by parathyroid hormone in tissue culture and in thyroparathyroidectomized rats. *Calcif Tiss Res* 1970; 6: 183-196.
49. Sahni M, Guenther HL, Fleisch H, et al. Bisphosphonates act on rat bone resorption through the mediation of osteoblasts. *J Clin Invest* 1993; 91: 2004-2011.
50. Sato M, Grasser W. Effects of bisphosphonates on isolated rat osteoclasts as examined by reflected light microscopy. *J Bone Miner Res* 1990; 5: 31-40.

51. Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD, Golub E, Rodan GA. Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. *J Clin Invest* 1991; 88: 2095-2105.
52. Schenk R, Eggli P, Fleisch H, Rosini S. Quantitative morphometric evaluation of the inhibitory activity of new aminobisphosphonates on bone resorption in the rat. *Calcif Tissue Int* 1986; 38: 342-349.
53. Schenk R, Merz WA, Mühlbauer R, Russell RGG, Fleisch H. Effect of ethane-1-hydroxy-1,1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl_2MDP) on the calcification and resorption of cartilage and bone in the tibial epiphysis and metaphysis of rats. *Calcif Tiss Res* 1973; 11: 196-214.
54. Schmidt A, Rutledge SJ, Endo N, et al. Protein-tyrosine phosphatase activity regulates osteoclast formation and function: inhibition by alendronate. *Proc Natl Acad Sci USA* 1996; 93: 3068-3073.
55. Shinoda H, Adamek G, Felix R, Fleisch H, Schenk R, Hagan P. Structure-activity relationships of various bisphosphonates. *Calcif Tissue Int* 1983; 35: 87-99.
56. Storm T, Steiniche T, Thamsborg G, Melsen F. Changes in bone histomorphometry after long-term treatment with intermittent, cyclic etidronate for postmenopausal osteoporosis. *J Bone Miner Res* 1993; 8: 199-208.
57. Toolan BC, Shea M, Myers ER, et al. Effects of 4-amino-1-hydroxybutylidene bisphosphonate on bone biomechanics in rats. *J Bone Miner Res* 1992; 7: 1399-1406.
58. Trechsel U, Schenk R, Bonjour J-P, Russell RGG, Fleisch H. Relation between bone mineralization, Ca absorption, and plasma Ca in phosphonate-treated rats. *Am J Phyiol* 1977; 232: E298-E305.
59. Trechsel U, Stutzer A, Fleisch H. Hypercalcemia induced with an retinoid in thyroparathyroidectomized rats. A new model to study bone resorption in vivo. *J Clin Invest* 1987; 80: 1679-1686.
60. Tsuchimoto M, Azuma Y, Higuchi O, et al. Alendronate modulates osteogenesis of human osteoblastic cells in vitro. *Jpn J Pharmacol* 1994; 66: 25-33.
61. Vitté C, Fleisch H, Guenther HL. Osteoblasts mediate the bisphosphonate inhibition on bone resorption through synthesis of an osteoclast-inhibiting activity. *J Bone Min Res* 1994; 9 (Suppl.1): S142, Abstract 87.
62. Von Baeyer H, Hofmann KA. Acetodiphosphorige Säure. *Ber Dtsch Chem Ges* 1897; 30: 1973-1978.
63. Wiedmer WH, Zbinden AM, Trechsel U, Fleisch H. Ultrafiltrability and chromatographic properties of pyrophosphate, 1-hydroxyethylidene-1,1-bisphosphonate, and dichloromethylenebisphosphonate in aqueous buffers and in human plasma. *Calcif Tissue Int* 1983; 35: 397-400.
64. Wronski TJ, Dann LM, Qi H, Yen CF. Skeletal effects of withdrawal of estrogen and diphosphonate treatment in ovariectomized rats. *Calcif Tissue Int* 1993; 53: 210-216.
65. Zimolo Z, Wesolowski G, Rodan GA. Acid extrusion is induced by osteoclast attachment to bone: inhibition by alendronate and calcitonin. *J Clin Invest* 1995; 96: 2277-2283.