0163-769X/98/$03.00/0
Endocrine Reviews 19(1): 80–100
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Bisphosphonates: Mechanisms of Action

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I. Introduction

The bisphosphonates have been known to chemists since the middle of the 19th century, when the first synthesis occurred in 1865 in Germany (1). Etidronate, the first bisphosphonate to be used to treat a human disease (2), was synthesized exactly 100 yr ago (3). Bisphosphonates were used in industry, mainly as corrosion inhibitors or as complexing agents in the textile, fertilizer, and oil industries. Their ability to inhibit calcium carbonate precipitation, similar to polyphosphates, was put to good use in the prevention of scaling (4). Only in the past three decades have bisphosphonates been developed as drugs for use in various diseases of bone, tooth, and calcium metabolism.

Our knowledge of the biological characteristics of bisphosphonates dates back 30 yr. The first report was done by the author's group and published in 1968 (5). The concept was derived from our earlier studies on inorganic pyrophosphate. We had found that plasma and urine contained compounds that inhibit calcium phosphate precipitation and that part of this inhibitory activity was due to inorganic pyrophosphate, a compound that had not been described previously in the scientific literature (6). Pyrophosphate was then shown to impair *in vitro* the formation and dissolution of calcium phosphate crystals. This effect was therefore similar to that on calcium carbonate and, for this reason, had been used in washing powders. Since pyrophosphate was able to inhibit ectopic calcification *in vivo*, it was suggested that it might act as a physiological regulator of calcification and perhaps also of decalcification *in vivo*, its local concentration being determined by the activity of local pyrophosphatases (7).

Because of its failure to act when given orally and its rapid hydrolysis when given parenterally, pyrophosphate was used therapeutically only in scintigraphy and against dental calculus. This prompted us to search for analogs that showed similar physicochemical activity but resisted enzymatic hydrolysis and, therefore, would not be degraded metabolically. The bisphosphonates fulfilled these conditions.

This review will deal with the mechanisms of action of these compounds. *In vitro* results, as well as results both in animals and humans, will be integrated in an attempt to deduce the current state of the art. Various reviews have been published recently on bisphosphonates and may be consulted also for information on other aspects (8–14). Since the literature in this field is plentiful, selective citation was necessary. Priority is given to papers dealing with the mechanisms of action. Since many papers often deal with the same finding, in most cases only the first ones are quoted. Subsequent papers are quoted only if they convey new knowledge.

II. Chemistry

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 of pyrophosphate, containing an oxygen instead of a carbon atom (Fig. 1). In the literature these compounds are usually called bisphosphonates. Although this is not entirely correct since nongeminal bisphosphonates are also bisphosphonates, we shall nevertheless adopt this nomenclature for simplicity's sake.

The P-C-P structure allows a great number of possible variations, either by changing the two lateral chains on the carbon or by esterifying the phosphate groups. The bisphosphonates described in Fig. 2 have been investigated in humans with respect to their effects on bone. Six of them are commercially available today for treatment of bone disease (Fig. 2).

Each bisphosphonate has its own chemical, physicochemical, and biological characteristics, which implies that it is not possible to extrapolate from the results of one compound to others with respect to its actions.

III. Effects *in Vivo*

The bisphosphonates have two fundamental biological effects: inhibition of calcification, when given at high doses, and inhibition of bone resorption.

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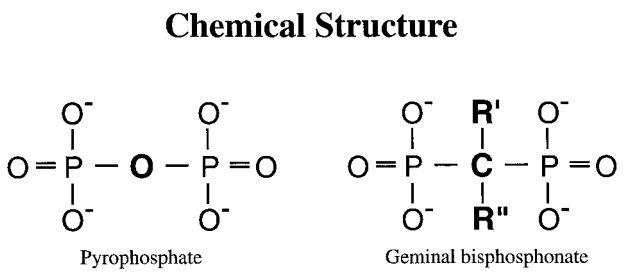

FIG. 1. Chemical structure of pyrophosphate and bisphosphonates.

A. Inhibition of calcification

The first rationale for the search for analogs of polyphosphates was to find compounds that would inhibit the formation of calcium phosphate salts without being destroyed by enzymes, therefore making them useful in treating diseases with ectopic mineralization. One possible application was to administer the compounds systemically in diseases such as atherosclerosis; another application was as an addition to toothpastes to fight against dental calculus.

1. Ectopic mineralization and ossification.

a. In animals: Bisphosphonates can efficiently inhibit ectopic calcification *in vivo*. Thus, among others, they prevent experimentally induced calcification of many soft tissues when given both parenterally and orally (15, 16). In contrast to pyrophosphate, which acts only when given parenterally, they are also active when administered orally. They decrease not only mineral deposits but also the accumulation of cholesterol, elastin, and collagen in the arteries (17, 18).

Bisphosphonates can also inhibit the calcification of bioprosthetic heart valves. Thus, etidronate administered subcutaneously inhibits the calcification of aortic valves implanted subcutaneously in rats (19). The bisphosphonate is also active when it is released locally from various matrices (20, 21). Certain results suggest that the bisphosphonates can be bound covalently to the valves (22). These results open an interesting field of application in heart surgery.

Bisphosphonates also decrease the formation of experimental urinary stones (23). Unfortunately, the dose has to be such that normal mineralization is impaired, as well.

As originally hypothesized, topical administration can lead to a decreased formation of dental calculus (24). This effect is currently used to prevent tartar formation in humans by the addition of bisphosphonates to toothpastes.

Finally, certain bisphosphonates also inhibit ectopic ossification when given systemically (25) or locally (26). It appears that the process is mainly an impairment of the calcification process because the deposition of matrix is not impaired, at least in the beginning.

b. In humans: One of the bisphosphonates, etidronate, has been used in humans to prevent ectopic calcification and ossification. Unfortunately, with respect to calcification, the results so far have been disappointing. In conditions such as scleroderma, dermatomyositis, and calcinosis universalis, results are inconclusive (27). In urolithiasis, the dose that might be effective is such that normal mineralization is inhibited (28). Better effects are seen with topical applications to prevent dental calculus (29, 30), and toothpastes containing bisphosphonates are marketed in some countries. More published reports are available in ectopic ossification, especially fibrodysplasia ossificans progressiva (31), and ossification after spinal cord injury, cranial trauma, and especially after total hip replacement (32, 33). However, the efficacy of etidronate has still not been proven beyond a doubt, although the results are promising (34).

2. Normal mineralization. The results cited above raised the hope that bisphosphonates might indeed be used clinically to inhibit various types of calcifications. Unfortunately, however, when administered in doses approximating those that inhibit soft tissue calcification, bisphosphonates can impair the mineralization of normal calcified tissues such as bone and cartilage (35–37) and, when given in higher amounts, also dentine (38), enamel (39, 40), and cementum (41). In the latter case, their administration can lead to a reduction of the extraction force.

While the different compounds vary greatly in their activity in bone resorption, they do not vary greatly in the inhibition of mineralization. For most species the effective daily dose is on the order of 5–20 mg of compound phosphorus per kg, administered parenterally. Interestingly, clodronate inhibits normal mineralization to a lesser degree than etidronate. The inhibition is eventually reversed after discontinuation of the drug (37). The inhibition of mineralization can lead to impaired fracture healing (42).

Since the inhibition is not corrected by 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ (43), it shows that the defect is not due to a decrease in this hormone. The decrease in calcitriol, which is sometimes observed when large amounts of etidronate are given (44, 45), and which is accompanied by a decrease in intestinal calcium absorption (46), is most probably secondary to the inhibition of mineralization. The decrease represents a homeostatic mechanism that adapts intestinal calcium absorption to the needs of the organism to maintain calcium homeostasis (47). When bisphosphonates are given in amounts small enough to decrease bone resorption without inhibiting mineralization, an increase in both plasma calcitriol and intestinal calcium absorption is observed (48).

Bisphosphonates also inhibit calcification of bone in humans when given in larger amounts (49–52) (see *Section VI*).

The propensity to inhibit the calcification of normal bone has hampered the therapeutic use of bisphosphonates in ectopic calcification.

B. Inhibition of bone resorption

Bisphosphonates can be very powerful inhibitors of bone resorption, their potency varying according to their structure. This was shown *in vitro* in cell and organ culture, as well as *in vivo* in both animals and humans. The effect is present in normal animals as well as in experimental conditions in which resorption is increased. Similarly, bone resorption is decreased in normal individuals as well as in patients afflicted with a series of conditions accompanied by increased bone resorption, such as Paget's disease, tumoral osteolysis, hyperparathyroidism, and osteoporosis.

1. Effects in vivo. Bisphosphonates inhibit bone resorption both in intact animals and in those with experimentally increased resorption.

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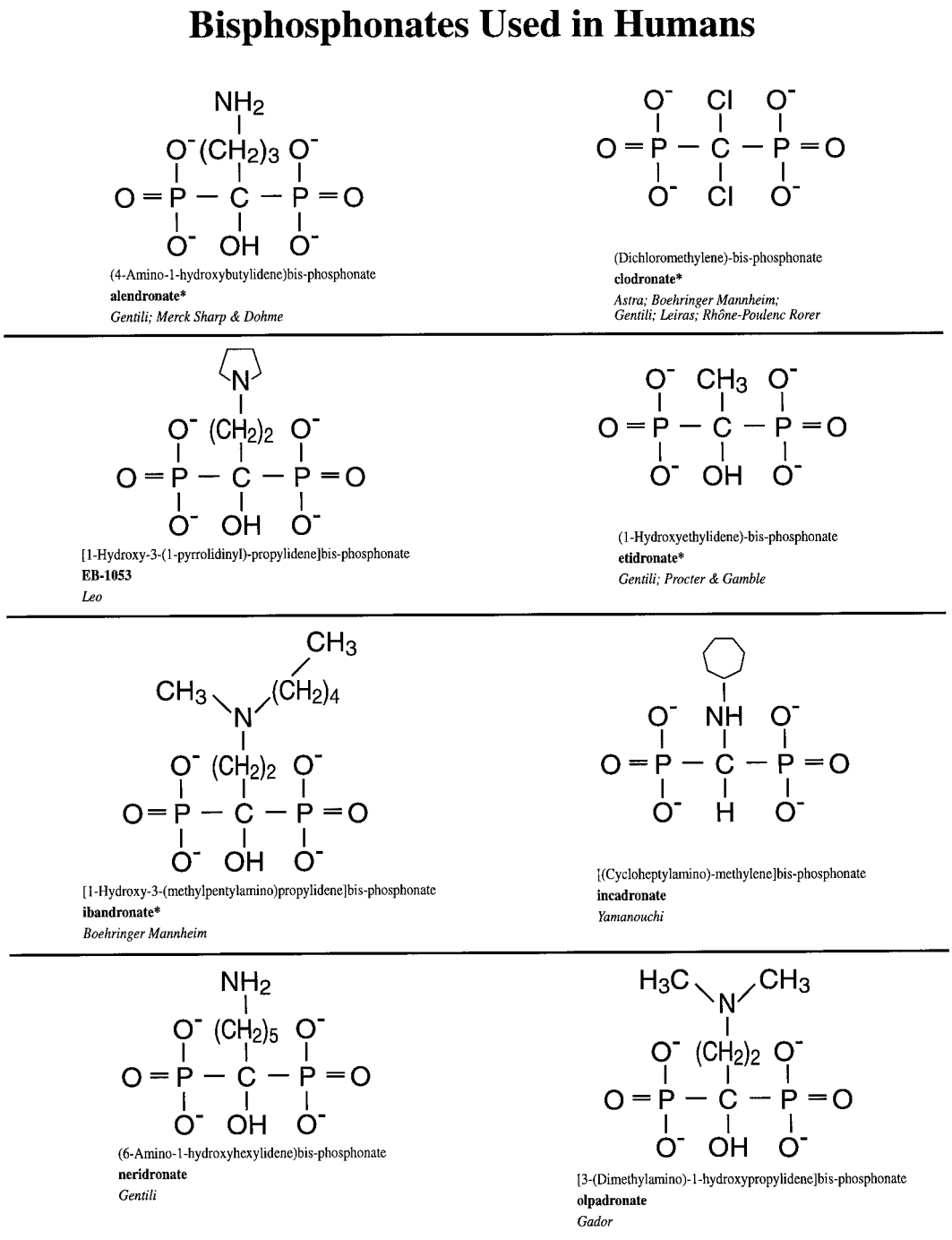

FIG. 2. Chemical structure of the bisphosphonates investigated for their effects in humans. *, Commercially available. [From H. Fleisch (14).]

a. Intact animals: In growing intact 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 (36). This is similar to observations in animals with congenital osteopetrosis (53). These various changes are all secondary to the inhibition of bone resorption. This effect is used as a model with which to study the potency of new compounds (54).

The inhibition of endogenous bone resorption has also been documented by ^{45}Ca kinetic studies (55, 56) and by markers of bone resorption (55). The effect occurs within 24–48 h (57) and is therefore slower than that of calcitonin.

In view of the accumulation of the bisphosphonates in bone, it is of great clinical interest that the inhibition of bone resorption reaches a certain steady level even when the compounds are given continuously (58). This level depends on the administered dose. This has also been described in humans (59). These results show that there is no accumulation of effect with time and suggest that the bisphosphonate buried in the bone is inactive, at least as long as it remains buried

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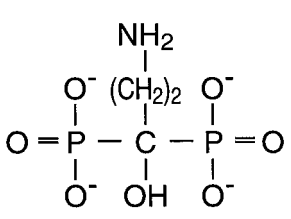
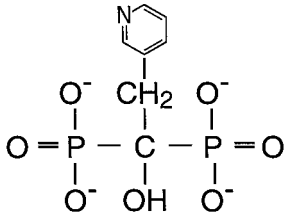
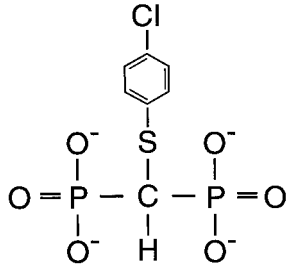


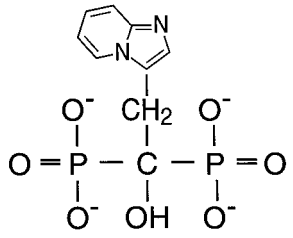
(3-Amino-1-hydroxypropylidene)bis-phosphonate
pamidronate*
Ciba-Geigy; Gador



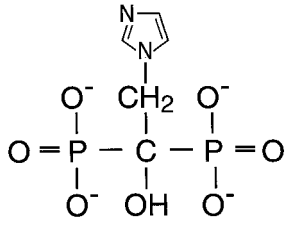
[1-Hydroxy-2-(3-pyridinyl)-ethylidene]bis-phosphonate
risedronate
Procter & Gamble



[[(4-Chlorophenyl)thio]-methylene]bis-phosphonate
tiludronate*
Sanofi



[1-Hydroxy-2-imidazo-(1,2-a) pyridin-3-ylethylidene]bis-phosphonate
YH 529
Yamanouchi–Hoechst



[1-Hydroxy-2-(1H-imidazole-1-yl)ethylidene]bis-phosphonate
zoledronate
Ciba-Geigy

FIG. 2. *Continued*

there. They also show that, at the therapeutic dosage, there is no danger of a continuous decrease in bone turnover in the long run, coupled with an increase in bone fragility, as seen in osteopetrosis.

The decrease in resorption is accompanied by an increase in calcium balance (55, 56) and in mineral content of bone. This is possible because of an increase in intestinal absorption of calcium (55, 56) consequent to an elevation of 1,25-$(OH)_2$ vitamin D. This increased balance is the reason for administering these compounds to humans suffering from osteoporosis. However, the increase is smaller than predicted, considering the dramatic decreases in bone resorption and bone formation (55, 56), possibly due to the so-called "coupling" between formation and resorption. This will be discussed in a later section.

Similar results are found in humans. Bisphosphonates decrease both resorption and formation, as described in numerous studies (for reviews, see Refs. 12 and 14).

b. Animals with experimentally increased resorption including osteoporosis: Bisphosphonates can also prevent experimentally induced increases in bone resorption. They impair resorption induced by agents such as PTH (60, 61), 1,25-$(OH)_2$ vitamin D, and retinoids. The effect on retinoid-induced hypercalcaemia has been used to develop a powerful and rapid screening assay for new compounds (62).

The bisphosphonates are also effective in preventing bone destruction in a number of disease models.

i. Osteoporosis. Many osteoporosis models have been investigated, including sciatic nerve section [which was the first model investigated (63)], spinal cord section, hypokinesis, ovariectomy (64, 65), orchidectomy (66), heparin, lactation (67), low calcium diet, and corticosteroids (68). All bisphosphonates investigated, *i.e.*, alendronate, clodronate, etidronate, ibandronate, incadronate, olpadronate, pamidronate, risedronate, tiludronate, and YH 529, have been effective.

Bisphosphonates also decrease bone loss and actually increase bone mineral density in humans with postmenopausal osteoporosis (69–74) and corticosteroid-induced bone loss (75). Alendronate and tiludronate also prevent bone loss in healthy postmenopausal women (76, 77).

The effect of bisphosphonates upon the mechanical properties of the skeleton has been addressed only recently. This issue is important since longlasting, strong inhibition of bone resorption can lead to increased bone fragility and, therefore, to fractures caused by an inability to replace old bone by

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young bone and to repair microcracks. Such an effect of bisphosphonates is present when very large amounts of bisphosphonates are administered to animals. Thus, mice given such a treatment from birth develop a radiological and morphological bone appearance similar to that seen in congenital osteopetrosis (53). Dogs develop an increase in fractures if given very large amounts of etidronate or clodronate over a year (37). In contrast, doses of risedronate 5 and 20 times the anticipated clinical dose did not induce any increase in microdamage of the bones of dogs, despite the fact that the activation frequency, an index of bone turnover, was decreased between 53% and 94% (78).

It is now clear that, if not given in excess, bisphosphonates improve biomechanical properties both in normal animals and in experimental models of osteoporosis. This is the case with alendronate, clodronate, etidronate, incadronate, neridronate, olpadronate, pamidronate, tiludronate, and YH 529. This effect is seen in various animals such as the rat, the chick, and the baboon (65, 79–82). Note, however, that the effect is more ambiguous with etidronate, since at higher doses it is obscured by an inhibition of mineralization.

Recent human data show that alendronate actually decreases the incidence of both vertebral and nonvertebral fractures (72, 83). However, it will always be prudent to administer a dose that does not induce too profound an inhibition of turnover. In treating osteoporosis, the general aim is to attain levels that correspond to those observed before the menopause. This is obtained, for example, with 10 mg daily of alendronate (59).

ii. Tumor bone disease. Bisphosphonates partially or entirely correct the increase in bone resorption in experimental tumor bone disease. Etidronate and clodronate inhibit the bone resorption induced by supernatants of tumor cultures *in vitro* (84, 85). *In vivo*, various bisphosphonates partially correct the hypercalcemia induced in rats by subcutaneously implanted Walker 256 carcinomas (86, 87) or Leydig tumors (88). For calciuria, the effect is generally more pronounced than for 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 (89), with bisphosphonates acting only on the former. Bone resorption secondary to actual tumor invasion is also retarded, as shown by numerous models using different tumor cells. The bisphosphonates shown to be active were, among others, clodronate, etidronate, incadronate, pamidronate, and risedronate (for review see Ref. 90). Of great clinical interest is the fact that not only osseous metastases but also tumor burden is decreased, at least with risedronate (91). On the other hand, an increase in the burden has been described with a different bisphosphonate and another type of cell (92). The mechanism of the decrease in tumor burden is still debated. The decrease may be due to the diminished release of growth factors that are present in bone matrix and may stimulate tumor cell growth during bone resorption (93). Another possibility would be less space in bone, which might prevent the tumor cells from developing.

In humans, bisphosphonates inhibit tumor-induced bone resorption, correct hypercalcemia, reduce pain, prevent development of new osteolytic lesions, prevent the occurrence of fractures and, consequently, improve the quality of life (94–99). They are now the treatment of choice in hypercalcemia of malignancy.

iii. Periodontal disease. Another interesting future use is in alveolar bone resorption. Bisphosphonates have been shown to decrease the bone destruction in various animal models (100–102).

2. Effects in organ and cell culture. Bisphosphonates block bone resorption induced by various means in organ culture (60, 61, 103, 104). For many years it was not possible to obtain a good correlation between the results obtained *in vitro* and those found *in vivo*. Recently, however, such a correlation was obtained using the mouse calvaria system (105).

An inhibition can also be found when the effect of isolated osteoclasts on various mineralized matrices is investigated *in vitro* (106–108). Under bisphosphonate treatment, the osteoclasts form fewer erosion cavities, which are of smaller size. However, only certain models show the same sequence of potency as that found *in vivo* (109).

3. Potency of various bisphosphonates on bone resorption. One of the aims of bisphosphonate research has been to develop compounds with a more powerful antiresorptive activity but without a higher inhibition of mineralization. This is possible since the activity of bisphosphonates on bone resorption varies greatly from compound to compound. Compounds have now been developed that are 5,000–10,000 times more powerful than etidronate in inhibiting bone resorption. The gradation of potency evaluated in the rat corresponds quite well with that found in humans (Table 1).

4. Structure-activity relationship. To date, no clear-cut relationship between structure and activity could be perceived. The length of the aliphatic carbon is important since activity increases up to a certain length and decreases thereafter. Adding a hydroxyl group to the carbon atom at position 1 increases potency (110). Derivatives with an amino group at the end of the side chain are very active. The first of these compounds to be described was pamidronate (58, 111). Again, the length of the side chain is relevant, the highest activity being found where there is a backbone of four carbons, as in alendronate (54). A primary amine is not necessary for this activity since dimethylation of the amino nitrogen of pamidronate, as seen in olpadronate, increases efficacy (112). Activity is still further increased when other groups are added to the nitrogen, as seen in the extremely active ibandronate (113). Cyclic geminal bisphosphonates are also very potent, especially those, such as risedronate, that contain a nitrogen atom in the ring. The most active compounds described so far, zoledronate (105) and YH 529, belong to this class. This intriguing effect of nitrogen is not yet explained. A three-dimensional structural requirement appears to be involved. Indeed, stereoisomers of the same chemical structure have shown a 10-fold difference in activity (114). This opens the possibility of a binding to some kind of "receptor," or "active" sites.

Until recently it was thought that only geminal compounds (*i.e.*, compounds with only one carbon between the two P atoms) were effective. In 1995 it was reported that longer chain compounds could be made effective both on the inhibition of calcification *in vitro* and *in vivo,* as well as on

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TABLE 1. Potency of the major bisphosphonates to inhibit bone resorption in the rat

~1×	~10×	~100×	>100–<1000×	>1000–<10,0000×	>10,000×
Etidronate	Clodronate	Pamidronate	Alendronate	Ibandronate	YH 529
	Tiludronate	Neridronate	EB-1053	Risedronate	Zoledronate
			Incadronate		
			Olpadronate		

[From H. Fleisch (14).]

bone resorption, if a keto group in the α-positions near the phosphoric functions was added (115). Again, as for the bisphosphonates, the chain length is important. These bisacylphosphonates might be of interest in the future.

C. Effects on bone formation

Until recently, bisphosphonates were considered not to affect bone formation directly but to increase bone balance merely by inhibiting bone resorption. However, new results suggest that this may not be entirely true. Morphological data on the basic structural unit suggest a possible increase in formation in the bone multicellular unit (BMU), implying that some stimulating effect on bone formation might be present (see *Section IV.B.1.*) (65, 116, 117).

It is noteworthy that incadronate administered at toxic doses orally for 13 weeks was found to produce intramembranous intramedullary bone formation (118). No explanation has yet been found for this unique phenomenon.

At the cellular level bisphosphonates have been shown to increase *in vitro* the proliferation of osteoblasts (119, 120) and cartilage cells (121), as well as the biosynthesis of collagen and osteocalcin by bone cells (119, 122, 123) and proteoglycans by cartilage cells (124). The effect on collagen may be partially due to impaired intracellular collagenolysis (125). Alendronate can increase colony formation of osteoblasts (119) and the formation of mineralized nodules in human cell cultures *in vitro*, a phenomenon that is accompanied by an increased formation of basic fibroblast growth factor (126). It has been suggested that some of these effects may be mediated through protein-tyrosine phosphatases (120).

Thus it is possible that bisphosphonates could, under certain circumstances, also act by increasing bone formation. This possibility, although far from being established, is of enough potential interest to deserve a thorough investigation.

D. Effects on noncalcified tissues

Bisphosphonates also have some effects *in vivo* that are not necessarily related to the effects on bone. Often, however, these effects occur after very large doses, so that any relevance to pharmacological doses is doubtful. The effects on the immune system are discussed in *Section IV.B.5.b*. Of possible clinical interest is an increase in plasma high-density lipoproteins. This, and the fact that bisphosphonates and phosphonosulfonates linked to an isoprene chain are potent inhibitors of squalene synthase and hence cholesterol-lowering agents in animals (127) may open some interesting new therapeutic applications for these drugs.

A clinically important effect, the mechanism of which is not yet understood, is their influence on mucosa. It has been known for a long time that bisphosphonates can induce gastrointestinal disturbances (128). These appeared to be more pronounced for the aminobisphosphonates. It is now known that pamidronate (129), as well as alendronate (130), can, when given orally, induce serious adverse esophageal effects such as esophagitis, erosions, and ulcerations.

IV. Mechanisms of Action

A. Calcification

The mechanism of the inhibition of both normal and ectopic mineralization is most likely due, in part if not entirely, to a physicochemical mechanism. 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* (15, 47, 131); therefore, the mechanism is likely to be a physicochemical one. It is of interest that, in contrast to what occurs in bone resorption, the bisphosphonate must be continuously present to exert this effect both *in vitro* (131) and *in vivo* (36, 132).

The physicochemical effects of most of the bisphosphonates are very similar to those of pyrophosphate. Thus, they inhibit the formation and aggregation of calcium phosphate crystals from clear solutions, even at very low concentrations (15), block the transformation of amorphous calcium phosphate into hydroxyapatite (133, 134), and delay the aggregation of apatite crystals (135).

Bisphosphonates also delay the dissolution of calcium phosphate crystals (60, 61, 136). This effect was one of the reasons for investigating the action of these compounds on bone resorption *in vivo*. While they indeed proved to be good inhibitors of bone resorption, the mechanism is now thought not to be physicochemical but rather biological.

All of these effects appear to be related to the marked affinity of these compounds for the surface of solid-phase calcium phosphate where they bind onto the calcium by chemisorption (137), presumably chiefly at screw dislocations and kink sites of growth, and then act as a crystal poison on both growth and dissolution. The binding can be of two types (138, 139): bidentate or tridentate. In bidentate binding, an oxygen atom from each phosphonate group binds onto a calcium of the hydroxyapatite. Clodronate is an example of this type of binding. Most of the bisphosphonates that are now used clinically are tridentate. They bind at a third location, such as the oxygen of a hydroxyl group on the central carbon. This tridentate binding displays a better binding strength, which explains why clodronate is relatively less bound. A nitrogen atom can take the place of the hydroxyl group, as in incadronate. There is a positive relation between the binding of various bisphosphonates and their inhibitory effect on crystallization (131), giving strong support to the

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theory that the inhibition of mineralization *in vivo* is due to a physicochemical mechanism.

To date, there is no indication that the bisphosphonates are incorporated into the crystal lattice of hydroxyapatite. They are, however, incorporated into the bone because the crystals, along with bisphosphonate, on their surface become trapped by new crystals formed on top of them.

Bisphosphonates also inhibit the formation (23, 140) and the aggregation (141) of calcium oxalate crystals. These effects on calcium phosphate and oxalate crystal formation raised the hope that bisphosphonates might be used to prevent urinary lithiasis. This proved not to be possible since the dose necessary to inhibit crystallization in urine also induces an inhibition of normal mineralization, leading to the development of osteomalacia (28).

While these results point to a physicochemical mechanism in the inhibition of calcification, an effect on matrix formation cannot be totally excluded. When etidronate is given in doses that produce mineralization defects, changes in glycosaminoglycan synthesis are seen in teeth (142) and growth plate cartilage (143). Furthermore, collagen synthesis seems to be effected in dentine (38, 144, 145) and heterotopic bone (25, 146). These changes, as well as those observed in arteries (17, 18), could be a consequence of the inhibition of mineralization. However, it is interesting that changes are seen also in nonmineralized tissues such as articular cartilage (147).

B. Bone resorption

First of all, it must be stressed that, while the effects on calcification are probably explained by a physicochemical mechanism on the crystals, this is not the case for bone resorption. The inhibition of bone resorption can actually be explained largely, if not entirely, by cellular mechanisms. The latter can be considered at three levels: tissue, cellular, and molecular. The effect may be directly on the osteoclasts and may be mediated, at least partially, by other cells such as osteoblastic lineage cells and macrophages.

1. Physical chemistry. The earliest hypothesis for the action of bisphosphonates on bone proposed physicochemical effects on mineral dissolution. Bisphosphonates, like pyrophosphate, do indeed inhibit mineral dissolution (7, 60, 61, 136). However, the concentrations of bisphosphonates required to inhibit bone resorption with the newer, more potent compounds are so low that they are unlikely to have a significant impact on mineral dissolution. Moreover, structure/activity studies on a large array of compounds showed no correlation between the inhibition of mineral dissolution *in vitro* and the pharmacological activity on bone resorption *in vitro* (131) or *in vivo* (110). It is therefore accepted by most investigators that the effect on bone resorption is essentially cellular.

2. Tissue level. At this level, the action of the active bisphosphonates appears to be the same for all, *i.e.*, a reduction in bone turnover. This is shown by a decrease in both bone resorption and bone formation, as assessed in animals as and humans by calcium-^{45} kinetics (55, 56), biochemical markers (59), and morphology (36, 65, 116, 117).

Under normal conditions, destroyed bone is replaced by bone formation. In adults this occurs mostly at the sites of remodeling in both the trabeculae and the cortex. The morphological dynamic unit of the turnover is the BMU. The remodeling process in this unit starts with the erosion of a certain amount of bone through osteoclasts on the surface of the trabeculae, as well as on the surface or the interior of the cortex. The resorption follows a linear path, forming a canal within the cortex and a trench on the surface. The destruction is followed by a refilling of the excavation by the osteoblasts within a tight temporal sequence. This explains why every decrease in resorption is accompanied by a secondary decrease in formation, since there is less need for a bone defect to be replenished. The final morphological entity is called the bone structural unit (BSU). It corresponds to an osteon within the cortex and has of late been termed a hemiosteon when it is at the surface of the bone (148). The total bone resorption and formation will therefore depend upon the number of BMUs present at any time which, in turn, will depend upon both the number of BMUs formed and the length of time they are active (for reviews, see Refs. 148–150).

Under normal conditions, the amount of bone formed in each BMU equals the amount destroyed, so that the balance is zero. In osteoporosis, however, a greater amount of bone is resorbed than formed, leading to a negative balance. Thus, while a change in turnover has no influence on the total calcium balance in normal people, there is a local negative bone balance in osteoporosis because more bone is destroyed than formed. Therefore, in this disease a decrease in turnover *per se* will slow down the total bone loss. This is why a high turnover after menopause, when such imbalance is present, is a good indicator for bone loss and the occurrence of osteoporosis in the future. This is also why all inhibitors of turnover, including bisphosphonates, will diminish bone loss in osteoporosis. In the case of bisphosphonates, it is probably the main mode of action in all types of osteoporosis. However, it must be stressed that there are conditions in which an increase in bone turnover is not necessarily accompanied by a negative balance. The growing animal is an obvious example, as well as certain cases of Paget's disease in humans.

In addition, the bisphosphonates also act at the individual BMU level by decreasing the depth of the resorption site (65, 116, 117). Since the amount of new bone formed in the BMU is not decreased, but possibly even increased (65, 116, 117), the local and consequently the whole body bone balance will be less negative or might even be positive.

The effect both on the general turnover and the local balance will lead to less trabecular thinning, a decreased number of trabecular perforations, a decreased reduction in connectivity (151), and a smaller erosion of the cortex, thus slowing down the decrease in bone strength and the occurrence of fractures.

Of crucial importance in the final effect is the behavior of the formation. As mentioned above, the total amount of bone formed is decreased because of the decrease in turnover, as shown by calcium^{45} kinetics, biochemical markers such as serum alkaline phosphatase and osteocalcin, and by a reduction in the bone formation surface assessed morphologically (55, 65, 116, 117). This reduction reflects reduced remodeling only. There is no evidence for reduced osteoblastic activity at individual bone formation sites, as judged by the

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amount of bone produced per unit time. On the contrary, the amount of bone formed at each individual basic structural unit (BSU), as measured by the thickness of the newly formed bone, is, if anything, increased (65, 116, 117). This effect is modest and needs to be confirmed. If present, however, such an effect could not be detected by any current technique measuring total bone formation in the body, such as biomechanical markers, since it would be obscured by the decrease in remodeling.

It is now generally accepted that bisphosphonates can lead to a positive calcium and bone balance, both in animals (55, 56) and in humans (69–77, 152). There are several explanations for this gain. One is inherent to bone turnover. Therefore, a decrease in bone resorption is not immediately followed by the diminution of formation, so that a temporary increase in balance through a reduction in the so-called remodeling space occurs. The second explanation is that, after the decrease in turnover, the new BSU formed will be remodeled later than it would be normally. It therefore has more time to finish the lengthy process of mineralization. This will lead to a higher calcium content and, therefore, a higher bone mineral density and content. However, it will not lead to an increase in actual bone mass, a fact that is often forgotten. Third, if the decrease in resorption depth at individual remodeling sites is not matched by a decrease in formation in the individual BMU, which seems to be the case, the local bone balance in the BMU will be positive. The last possibility is an increase in the amount formed at the level of the BMU (Fig. 3).

One of the important questions in connection with the clinical treatment of osteoporosis has been whether bone-forming substances would still be effective during bisphosphonate use. Except for one study (153), this seems to be the case for various stimulators of bone formation, such as PTH (154) and prostaglandins (155). Furthermore, bisphosphonates do prevent the loss of bone gained under the various stimulators of formation, which would otherwise occur (155–158).

Another question has been whether bisphosphonates could display an additive effect together with another inhibitor of bone resorption. One report suggests this to be the case with estrogen in humans (159).

3. Cellular level. There is now general agreement that the final target of bisphosphonate action is the osteoclast. Four mechanisms appear to be involved: 1) inhibition of osteoclast recruitment; 2) inhibition of osteoclastic adhesion; 3) shortening of the life span of osteoclasts; and 4) inhibition of osteoclast activity. The first three mechanisms will lead to a decrease in the number of osteoclasts, which is observed in humans and often, although not always, in animals. All four effects could be due either to a direct action on the osteoclast or its precursors or indirectly through action on cells that modulate the osteoclast.

1. Several bisphosphonates inhibit osteoclast differentiation in various culture systems of both cells (160) and bones (104, 112). Bisphosphonates are also powerful inhibitors of macrophage proliferation, cells that are of the same lineage as osteoclasts (161). In the hemopoietic series, the effect appears to be specific, or at least specially pronounced, for the mononuclear phagocyte lineage (162). Furthermore, the potency rank of bisphosphonates, when assessed *in vitro*, cor-

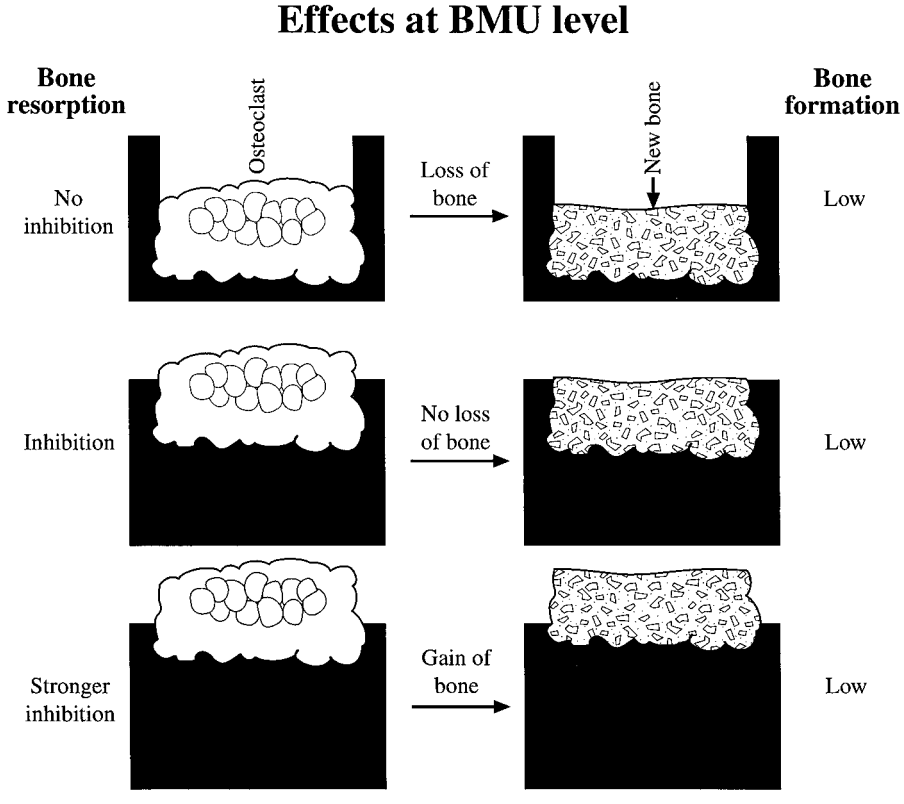

FIG. 3. Possible effect of bisphosphonates at the level of the individual BMU. [From H. Fleisch (14).]

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relates with effects *in vivo* only when systems are used that detect osteoclast recruitment and not activity alone (104, 112). Some experiments suggest that the effect occurs at the terminal step of the differentiation process (163). Other recent results (109, 164) also support the effect on differentiation. Thus, a correlation between the number of osteoclasts and osteoclastic cavity formation, on one hand, and the effect *in vivo*, on the other hand, occurs only if other cells, probably osteoblasts but not osteoclasts, are exposed to the bisphosphonates (see *Section* IV.B.5.a). Finally, when a system involving osteoclast differentiation is used (104, 112), the dose necessary to inhibit resorption is low only for aminobisphosphonates, but not for etidronate and clodronate, which are less powerful inhibitors of resorption. This suggests that two mechanisms may be operating, one on osteoclast recruitment and one with a direct effect on osteoclast activity.

2. The second possibility would be a decreased osteoclastic adhesion to the mineralized matrix. Whether this takes place is still uncertain since the results are ambiguous. One recent study reports such an effect (165). However, there is now excellent evidence that bisphosphonates can inhibit the adhesion of some cells, mainly tumor cells, *in vitro* (166).

3. The third possibility is a shortening of the lifespan of the osteoclast. It has been proposed that this might be due to a toxic effect, but the results were obtained at very high concentrations. Recently it was reported that bisphosphonates induce osteoclast programmed cell death (apoptosis), both *in vitro* and *in vivo*, and both in normal mice and in mice with increased bone resorption (167). The ranking of effectiveness of clodronate, pamidronate, and risedronate was the same as seen *in vivo*. The effect was not due to toxic cell death. Whether this is a direct effect on osteoclasts, or an indirect one through the effect on other cells, is not known. A similar effect occurs in macrophage-like cells *in vitro* and is nitric oxide independent (168).

4. The last possibility is an inhibition of osteoclast activity after the bisphosphonate has been taken up by the osteoclasts. Indeed, several facts suggest that the inhibition of recruitment is not the only mode of action of bisphosphonates *in vivo.* Thus, after bisphosphonate administration, the number of multinucleated osteoclasts on the bone surface often increases initially, despite a reduced bone resorption (36, 169, 170); however, the cells appear inactive (36). It is only later, after chronic administration, that the osteoclast number decreases. The cause for the initial increase is unknown. One possibility is that it could reflect a stimulation of osteoclast formation to compensate for the decrease in osteoclast activity.

A direct effect on the osteoclasts is supported by the finding that, under bisphosphonates, osteoclasts can show changes in morphology both *in vitro* (107, 170) and *in vivo* (36, 132, 169). These include changes in the cytoskeleton, especially actin (107, 171, 172) and vinculin (172), and the ruffled border (132, 169, 173). One study (171) showed that the morphological changes occurred only when the cells were actively resorbing the calcified matrix, or if the bisphosphonate was injected into the cells. No changes occurred when the osteoclasts were not active, showing that they have to be taken up with the resorbed mineral. As mentioned earlier, bisphosphonates inhibit the formation of resorption cavities by isolated osteoclasts deposited on calcified matrices *in vitro* (106–108). A direct action on osteoclasts is also supported by the fact that, under certain conditions, bisphosphonates can enter cells (174), particularly those of the macrophage lineage. The concentration of the bisphosphonate can also attain very high values under the osteoclasts, probably 100 μM or more, partly because they deposit preferentially under these cells (173, 175) and are then released from the mineral at the acid pH prevailing at this location.

4. Molecular level. The events leading to either osteoclast inactivation or diminished osteoclast formation by bisphosphonates have not yet been fully elucidated. It may be worth introducing this section by reiterating some general facts.

The circulating levels of pharmacologically active bisphosphonates are usually extremely low. This implies that uniform circulating levels are not necessary for continuous activity. This is supported by the fact that a single administration of these compounds can lead to a sustained inhibition of bone resorption which, *e.g.*, in patients with Paget's disease, can last over years. This suggests either that some cells are affected over a long time or, more likely, that the bisphosphonate taken up by the bone is released in very low amounts over time at areas of high turnover, thus affecting resorption locally. The latter would explain the high efficacy of these compounds in diseases with focal resorption, such as Paget's disease or metastases.

The other interesting fact is the low concentrations necessary for activity, which suggests either some sort of "receptor" or some cellular binding site, which induces a cellular transduction mechanism. Until now no such active receptor or binding site has been identified. However, the fact that osteoblasts exposed for only 5 min to very low concentrations of bisphosphonates are being stimulated into augmenting the release of an osteoblast recruitment inhibitor (100, 154) speaks in favor of their presence as a linking site. Since bisphosphonates enter the cell via fluid pinocytosis or adsorptive pinocytosis, the latter could be within the cell and might be an enzyme, a pump, or some other intracellular protein involved in the signaling cascade.

It has long been known that bisphosphonates decrease acid production of various cells (121) and of calvaria (176). In 1990, it was reported that bisphosphonates decrease the proton accumulation and the protein synthesis by osteoclasts *in vitro* (177). More recently, bisphosphonates were shown to decrease the extrusion of acid through a sodium-independent mechanism by true osteoclasts (178). Possibly part of this effect is due to the decrease of the proton transport by the vacuolar-type proton ATPase, which is inhibited by tiludronate, but surprisingly not by other bisphosphonates (179). However, until now no correlation between the effect *in vitro* on acid production and *in vivo* on bone resorption was evident. Some bisphosphonates, such as pamidronate or long-chain bisphosphonates, actually increase lactic acid production, possibly due to a toxic action (110, 180).

Various bisphosphonates, especially clodronate, inhibit lysosomal enzymes *in vitro* (181), in cultured calvaria (176, 182), or *in vivo* (180). Certain bisphosphonates, such as clodronate and etidronate, also inhibit prostaglandin synthesis by bone cells or calvaria, both *in vitro* and *in vivo* (183, 184). Since

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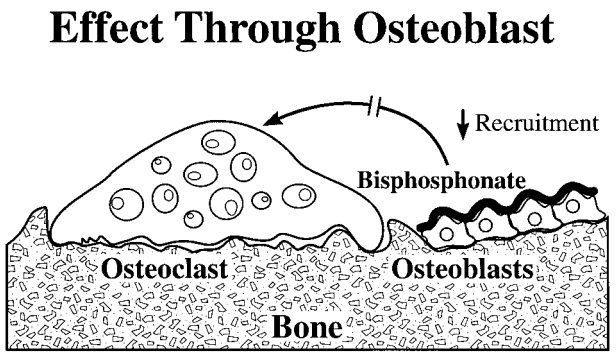

FIG. 4. Indirect effect of the bisphosphonates on the osteoclasts mediated by the osteoblasts. [From H. Fleisch (14).]

prostaglandins are involved in bone resorption, this inhibition may play a role in the resorption process.

Some data indicate that still other mechanisms may come into play. Thus, both in osteoporosis and in Paget's disease, bisphosphonates induce a decrease in urinary cross-links. This reflects the decrease in bone resorption. Surprisingly, in opposition to what occurs with estrogens, the effect is almost solely on peptide-bound collagen cross-links and not on free cross-links (185). This suggests that the bisphosphonates might influence the degradation process of collagen.

In view of the homology between pyrophosphate and bisphosphonates, various enzymes involving pyrophosphate or ATP have been examined. Phosphatases and pyrophosphatases were influenced only at relatively high concentrations (181, 186) or not influenced at all (187). However, PTPϵ, a protein-tyrosine phosphatase present in osteoclasts, is inhibited *in vitro* by alendronate with an IC_{50} of only 3 μM, while etidronate is active at 2 μM (187). Another protein-tyrosine phosphatase, PTPσ, which is present both in osteoclasts and osteoblasts, is also inhibited by alendronate and etidronate with an IC_{50} of 0.5 μM and 0.2 μM, respectively (120). Other protein-tyrosine phosphatases such as CD45 are also inhibited. These effects might be relevant since protein-tyrosine phosphorylation is important in the signal transduction pathways that control cell growth, differentiation, and activity. Furthermore, not only the bisphosphonates but also orthovanadate and phenylarsine oxide inhibit PTPs at very low concentrations and inhibit the formation of osteoclasts *in vitro* (187). Unfortunately, the potency to inhibit the PTPs of various bisphosphonates tested so far has no relationship to their pharmacological potency, since alendronate is about 1000 times more effective than etidronate on bone resorption *in vivo,* while their potency *in vitro* was of similar magnitude.

It was shown recently that various bisphosphonates, excluding clodronate, inhibit posttranslational modification of proteins, including the GTP-binding protein Ras, with farnesyl or geranylgeranyl isoprenoid groups in J774 macrophages. Furthermore, alendronate-induced apoptosis could be prevented in these cells by farnesylpyrophosphate or geranylgeranylpyrophosphate (M. J. Rogers, S. P. Luckman, F. P. Coxon, and R. G. G. Russell, submitted). This suggests that at least some bisphosphonates cause apoptosis through a mechanism involving prenylation of proteins. Whether this is true for osteoclasts must still be proven.

Another interesting observation is that both macrophage-like cells and human MG63 osteosarcoma cells metabolize primary clodronate to a nonhydrolysable ATP analog, adenosine 5′-(β,γ-dichloromethylene)triphosphate (189). This is not the case for other bisphosphonates. It has been suggested, therefore, that clodronate might act through this mechanism to induce apoptosis and necrotic cell death and therefore to inhibit bone resorption.

One of the conclusions based on the various biochemical results is that no single individual mechanism shows a good correlation with the potency *in vivo* when different bisphosphonates of various potencies are investigated. This suggests that, if any of the above mechanisms is relevant for bone resorption, it is not relevant for all bisphosphonates.

The various cellular modes of action are summarized in Table 2.

5. Effect through other cells. It appears more and more likely that the inhibitory effect is partly mediated through other cells, *e.g.*, one of the osteoblast-lineage cell.

a. Osteoblast-lineage cells: It is now generally accepted that cells of osteoblastic lineage control the recruitment and activity of osteoclasts under physiological and pathological conditions. This control was proposed to be due to the production of an as yet unknown activity, generated by osteoblast-lineage cells, and modulating bone resorption (190), and this modulation was thought to be an activation of resorption (191–194).

It has been shown that bisphosphonates may also act through the modulation of the osteoclast-osteoblast interrelation. It has been known for quite some time that, when assessed *in vitro*, various bisphosphonates can inhibit the destruction of the mineralized matrix, but that all those tested have a similar activity despite the fact that *in vivo* their antiresorbing effect varies from 1 to 1000 (107). This result suggests that the conditions created did not represent those operating *in vivo*. It was then discovered that this lack of correlation is only present when the bisphosphonates are added to the mineral before the osteoclasts, but not when the cell population containing the osteoclasts added to the matrix are treated for a time as short as 5 min, at concentrations as low as 10^{-11} M, before allowing them to adhere to the ivory (109). When doing this, five different bisphosphonates with potencies ranging from 1 to 10,000 showed a stringent correlation between the results *in vitro* and *in vivo* (109). Therefore, the best conditions are not when the bisphosphonates are on the mineral, as a direct effect on osteoclasts would imply, but when they are in contact with the cells.

TABLE 2. Possible biochemical action of bisphosphonates on the osteoclast

Binding to apatite crystals
Local release during bone resorption
Preferential accumulation under osteoclasts
↓
Decrease in osteoclast activity
−Altered cytoskeleton
−Ruffled border ↓
−Acid extrusion ↓
−Enzyme activity ↓
Decrease in osteoclast number
Apoptosis ↑

[From H. Fleisch (14).]

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This effect appears to be due to the osteoblasts present in the unpurified osteoclastic cell population. Thus, pretreating pure osteoblastic cell populations for 5 min with the bisphosphonates alendronate and ibandronate, and then coculturing the osteoblasts with the osteoclasts, prevented the usual increase in resorption (109). In contrast, adding osteoblastic cells to osteoclasts pretreated with the bisphosphonate had no effect. This result is supported by previous findings that, when assayed in a coculture of bone and osteoclast precursors, the bisphosphonates do not act directly on the precursors, but need the presence of a cell in the bone (104).

The inhibitory effect is not due to a decrease in the osteoclast-stimulating activity, but to the synthesis by the osteoblasts into the culture medium of an inhibitor of osteoclastic resorption. The latter is labile to heat and proteinase and has a molecular mass of approximately 3–4 kDa (164). The inhibitor has not been characterized, so that it is not possible to speculate as to what family it belongs.

The resorption cavities are reduced parallel to the reduction of the number of tartrate-resistant acid phosphatase-positive multi- and mononuclear cells, which are thought to be osteoclasts and their precursors. In contrast, the mean area resorbed by cavity remains unchanged, suggesting that the inhibitor affects osteoclast formation but not osteoclast activity (164). Other cells such as fibroblasts and preosteoblasts do not produce such an inhibitor. The question of which cells of the osteoblastic lineage are able to mediate this effect has not yet been answered. Recently, it has been postulated that lining cells play a role in the osteoblast-osteoclast relation (195) (Fig. 4).

It is interesting to note that 17β-estradiol also stimulates the synthesis of an osteoblast-derived osteoclastic inhibitor (196). However, this inhibitor appears to be different from that induced by bisphosphonate since it is entirely matrix-associated and, unlike the latter, does not go into the supernatant. This mechanism through the osteoblasts has been confirmed by various groups. Thus, pretreatment of UMR-106 osteoblast-like cells with bisphosphonates also induced a decrease in resorption cavities when they were cocultured with osteoclasts (197). The same result occurred when the osteoclasts were treated with the supernatant of treated UMR cells. The only difference from the above mentioned studies was that no effect was seen on the number of TRAP-positive cells, *i.e.*, of osteoclasts and their precursors, so that the effect was thought to be on osteoclast activity and not formation (197). Of interest is the finding that with ibandronate, the inhibitory factor was secreted into the supernatant, while with clodronate it remained attached to, or within, the osteoblast. In another study, incadronate also led osteoblasts to secrete an inhibitor, again of osteoclast formation, into the supernatant (198).

b. Cells of the mononuclear phagocyte and immune systems: The other possible candidates are the cells of the mononuclear phagocyte system and of the immune system. Since they produce a variety of bone-resorbing cytokines, it is possible that they may play a role in the cascade involved in the inhibition of bone resorption induced by a bisphosphonate.

There are numerous reports on the effect of bisphosphonates on these cells, both *in vitro* and *in vivo*. Unfortunately, these studies are often performed with high concentrations, so that the described effects might be secondary only to a toxic action. Low concentrations often give an effect contrary to that of higher concentrations, which might also reflect toxicity. Thus it is not possible at this time to state whether or not they are implied in the inhibition of bone resorption by bisphosphonates.

i. In vitro. It seems clear that the cells of the mononuclear phagocytic lineage are specially sensitive to bisphosphonates since other marrow populations are either much less or not at all influenced, at least *in vitro* (161, 162). The multiplication (161, 162) as well as the activity (199, 200) are both decreased. In addition, bisphosphonates have been reported to depress accessory function of monocytes (111), inhibit the action of mitogens on mononuclear function and on the lymphoblastic response (201), influence the effect of antilymphocyte serum on T lymphocytes (202), and inhibit migratory activities of macrophages (200). They also inhibit the proliferation of human peripheral blood mononuclear cells induced by various means. It has been suggested that this effect is mediated by the antigen-presenting cells (203).

With respect to cytokine production, clodronate inhibits lipopolysaccharide-induced interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNFα) production by a macrophage-like cell line (RAW 264) (204, 205). Alendronate inhibits, in a dose-dependent fashion, the production of these three cytokines by activated human monocytes (203). Pamidronate, however, increases the production of IL-6 (205). Clodronate and pamidronate, but not alendronate, also decrease the production of nitric oxide and the expression of inducible nitric oxide synthase in the RAW 264 cells (206). When clodronate is encapsulated into liposomes, its effect is increased while that of pamidronate is decreased.

ii. In vivo. The following effects on the immune system have been described: decrease in the formation of antibody-secreting cells and impaired delayed and immediate hypersensitivity (207); inhibition of passive cutaneous anaphylaxis (208); atrophy of the thymus (209); disappearance of certain thymus-dependent macrophages (210); disappearance of natural killer cells (211); and diminished response of the T lymphocytes to mitogens (209) in newborn mice. All these effects were obtained at very high dosages, some of which led to an osteopetrotic condition, so that the relevance to what occurs with clinical regimens is far from being proven. Indeed, none of these effects have been seen in humans.

The sensitivity of macrophages to bisphosphonates, especially to clodronate, has been made use of to selectively destroy macrophages *in vivo*. Thus, if bisphosphonates are administered encapsulated in liposomes, they are taken up by the macrophages mostly in the spleen and the liver, and the macrophages are then destroyed within 2 days (212). This technique has been used to study repopulating kinetics of macrophages and the role of macrophages in the organism.

An effect on macrophages, or possibly on other cells, might be the explanation for the acute phase response in humans. Thus, some patients who receive an amino-bisphosphonate intravenously for the first time show a transient pyrexia of 1–2 degrees C, sometimes more, accompanied by flu-like symptoms (111, 213). This episode is accompanied by a decrease in peripheral lymphocytes, especially the CD3+ T cells (214), an increase in C-reactive protein, and

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a decrease in serum zinc. Interestingly, this reaction occurs only once in a lifetime, even if the treatment is discontinued and restarted later. This raises the possibility, among others, that a specific cell population involved in the development of the acute phase reaction is influenced over longer periods. Recently, the pyrexia was shown to be accompanied by an increase in circulating IL-6 bioactivity (215). Furthermore, olpadronate but not clodronate stimulated the release *in vitro* of IL-6 from fetal mouse explants. In addition to IL-6, TNFα is also increased in the blood after treatment with pamidronate but not clodronate (216). The effect is not seen with etidronate, clodronate, or tiludronate. It is not known why only compounds that are potent inhibitors of bone resorption and contain a nitrogen atom in their structure show this effect.

Of clinical interest is that some bisphosphonates, including etidronate, clodronate, tiludronate, risedronate, and zoledronate, inhibit local bone and cartilage resorption, preserve the joint architecture, and decrease the inflammatory reaction in experimental arthritis induced by Freund's adjuvant, carrageenin and, to a smaller extent, collagen (217–221). The effect on the joints is especially pronounced when the bisphosphonates are encapsulated in liposomes (222, 223). The fact that not only bone resorption, but also the inflammatory reaction in the joint and in the paw itself, is diminished (223, 224) suggests that mechanisms other than those in bone, possibly involving the mononuclear phagocyte system, are operating. These results open the exciting possibility of using bisphosphonates in inflammatory arthritis, given either systemically or locally, possibly encapsulated in liposomes.

c. Tumor cells: As described in *Section III.B.1.b.ii*, bisphosphonates inhibit the bone resorption induced by various tumors both in animals (84–88, 91, 94) and in humans (96–99, 225–227). This is generally explained by the inhibition of bone resorption. The inhibited development of metastases can have various causes. One is that, since less bone has been destroyed, the place for tumoral expansion is limited. Another explanation is that, as a consequence to a decrease in bone resorption, the release of matrix or osteoclastic cytokines that would stimulate the multiplication of tumor cells may be decreased (91). In contrast, the bisphosphonates do not seem to inhibit directly the multiplication of tumor cells. Furthermore, there is now excellent evidence that bisphosphonates can inhibit the adhesion of tumor cells *in vitro* (166). The effect is specific for mineralized matrices, and the potency of various bisphosphonates is well correlated with the potency to inhibit bone resorption *in vivo*. It might explain in part the bisphosphonate-induced decrease in the development of tumor burden in animals (91).

C. Other effects

A great number of other cellular or biochemical effects have been described. They are confusing and can sometimes go in opposite directions with different compounds, or even with the same compound at different concentrations. With one or two exceptions, there is no indication that they are involved in bone resorption, and those most likely to play a role in the inhibition of bone resorption have been described earlier in this article. These other effects include the following: increase of fatty acid oxidation (228) and amino acid oxidation (180); stimulation of the citric acid cycle (180); increase in cellular content of glycogen (229); increase in production of alkaline phosphatase (230); inhibition of the 1,25-$(OH)_2D_3$-induced production of osteocalcin *in vivo* (231); contradictory effects on cAMP production (232, 233); decrease or increase in cellular multiplication (121, 234); inhibition of DNA polymerase (235); and inhibition of amoebal phosphofructokinase (236). A few results point to an effect on cellular calcium handling, *e.g.*, reduced release of calcium from kidney mitochondria *in vitro* (237) and increase in calcium of mitochondria *in vivo* (238); inhibition *in vitro* of calcium-induced contraction of smooth muscle, possibly through inhibition of intracellular Ca mobilization and influx of extracellular Ca (239); protection of the kidney from ischemic damage, possibly by preventing intracellular Ca accumulation (240). Considering this, it is interesting that nongeminal bisphosphonates act in a manner similar to Ca channel blockers (241). Finally, squalene synthase is inhibited (127).

It is interesting that bisphosphonates inhibit the growth of the slime mold amoeba *Dictyostelium discoideum,* and that some of them can form nonhydrolyzable methylene analogs of ATP (242, 243). The effect on growth of these organisms is of interest because of the presence of a remarkable correlation with a great number of different bisphosphonates between the effects found on this system using the growth of a slime mold and the bone resorption *in vivo* (244, 245). It suggests that this system might give us further insight in what occurs in bone resorption, which is supported by the fact that human cells can also perform such a transformation (189).

V. Pharmacokinetics

Bisphosphonates can enter mammalian cells. This has been confirmed by studies *in vitro* both for etidronate and clodronate (121, 174). The cellular uptake is mostly in the cytosol, and the concentration expressed in terms of cellular water can be several fold higher than in the medium (174). Cells with phagocytic properties display special avidity if the compounds are bound to apatite crystals (199).

Nevertheless, the bisphosphonates have a very low bioavailability, from a few percent for clodronate, etidronate, and tiludronate, which are given in larger amounts, to below 1% for the newer ones, which are given in low quantities. This is partly explained by their low lipophilicity, which hampers transcellular transport, and their high negative charge, which hampers paracellular transport. Furthermore, they are probably partly in an insoluble form in the gut, due to chelation to calcium. It is thought that the absorption in the intestine follows mainly a paracellular route (246). The latter is under the influence of calcium, which tightens the junctional complex. This explains why the administration of EDTA, a strong calcium chelator, increases the absorption of bisphosphonate (247) and why high doses of bisphosphonates, which also chelate calcium, will lead to an increase in

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their own absorption (248). Why a higher intestinal pH increases absorption while orange juice and coffee decrease it (249) is not known.

Some uncertainty still exists as to the state of bisphosphonates in the circulation. They are indeed only partially ultrafilterable in aqueous solutions as well as in plasma (250), possibly because of the formation of polynuclear aggregate complexes (251–253). In plasma they are bound to proteins, whereby this binding varies between compounds and between animals (254). The binding is pH and calcium dependent, whereby calcium and increasing pH augment it (255). There are also displacers of the binding in the plasma of, for instance, the dog (254). The role this binding could have on the action and the pharmacokinetics of bisphosphonates has never been investigated despite the fact that it may be conspicuous. For example, the assumption that bisphosphonates are not actively secreted in the kidney is probably wrong. Indeed, most renal studies were not corrected for the binding so that the filtered load was overestimated. If a correction is done, the results point to a secretory mechanism (256, 257).

Once in the blood, bisphosphonates disappear very rapidly, mostly to bone (258). This might be explained by the fact that they are characterized by a rapid and strong binding to the hydroxyapatite crystals (137). The rate of entry into bone is very fast, similar to that of calcium and phosphate. It has been calculated that the bone clearance is compatible with a complete extraction from the skeleton after the first passage (258), so that skeletal uptake might be determined above all by the vascularization of the bone. Consequently, soft tissues are exposed to these compounds for only short periods, explaining their bone-specific effects and their low toxicity.

The various bisphosphonates display some differences in their affinity for the hydroxyapatite surface. This reflects itself in the binding of bisphosphonates to bone *in vivo*. Thus, at least 50% of most of the hydroxylbisphosphonates distribute themselves to bone (259), whereas in the case of clodronate (260, 261) it is only about 20–40%. Their preferred location in the skeleton is bone with a high turnover, namely trabecular bone.

The binding of polyphosphates and bisphosphonates to calcified tissues is the basis for the use of these compounds as skeletal markers in nuclear medicine when linked to ^{99m}technetium. However, it is important to note that the handling of the technetium-labeled compounds is not identical with that of the bisphosphonates (262), so that caution must be given in extrapolating data from one to the other.

It was generally thought that the bisphosphonates deposit in those locations within the bone where new bone is formed. Recently, however, they were found to deposit under the osteoclasts as well (173). The distribution of the amount deposited at bone formation and bone resorption sites depends upon the amount of bisphosphonate administered (263). When small amounts are given, they deposit mostly under the osteoclasts while larger amounts go to both bone-forming and bone-resorbing sites. This would explain the results with ^{99m}technetium-labeled compounds, thought to go to formation sites, since larger amounts are usually injected. However, the fact that the erosion locations seen in multiple myeloma do not take up any visible radioactive ^{99m}technetium-labeled bisphosphonates has not yet been explained.

The fact that bisphosphonates are targeted to bone may be used in the future to administer drugs to the skeleton. Initial results with methotrexate in rats are encouraging (264).

Usually bisphosphonates do not deposit in soft tissues. However, some of them, especially pamidronate, can at times deposit in other organs such as the stomach (265), liver, and spleen (266–268), the deposition being proportionally greater when large amounts of compounds are given. Part of this extraosseous deposition appears to be due to the formation of complexes with iron (hemolysis) and calcium because of too high and too rapid an intravenous injection. The insoluble aggregate is then phagocytized by the macrophages of the reticuloendothelial system. Thus, results obtained with large amounts of labeled compounds given rapidly intravenously must be interpreted with caution. The danger of too rapid an infusion of large amounts of bisphosphonate exists also in humans where this procedure has led to renal failure (269) because of the formation of insoluble calcium aggregates in the blood.

Once the bisphosphonates are buried in the skeleton, they will be released only when the bone is destroyed in the course of the turnover. The skeletal half-life of various bisphosphonates is between 3 months and 1 yr for mice and rats (266–268) and is much longer, sometimes more than 10 yr, for humans (270).

The bisphosphonates are not metabolized *in vivo*. This is due to the stability of their P-C-P bond to heat and most chemical reagents, as well as to their resistance to hydrolysis by the enzymes found in the body. To date, all the bisphosphonates investigated were excreted unaltered. However, it is quite possible in the future that some compounds will be metabolized in their side chain, especially in the gut, so that it cannot be generally stated that bisphosphonates are not metabolized *in vivo*.

VI. Animal Toxicology and Human Adverse Events

A. Animal toxicology

Published animal toxicological data are scant. Acute, subacute, and chronic administration of bisphosphonates has in general revealed little toxicity. This is explained by their rapid incorporation into calcified tissue and hence their short presence in the circulation.

Acute toxicity is mostly due to hypocalcemia, which is induced by the formation of complexes or aggregates with calcium, leading to a decrease in ionized calcium.

The nonacute, nonskeletal toxicity is usually manifested, as is the case with many phosphates and polyphosphate, first in the kidney (271, 272). This occurs, however, only at doses substantially larger than those administered in humans. At still higher doses, other organs can show cellular alterations. The mechanisms leading to these changes are not known. In the skeleton and in teeth an inhibition of normal mineralization occurs, as mentioned earlier, usually at parenteral doses of approximately 10 mg/kg daily (35–41). As discussed earlier, this inhibition is explained by a physicochemical impairment of crystal growth. Large doses of bisphos-

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phonates can inhibit mineralization to such a level, which by itself can lead to an increased fragility and fractures (37). Finally, very large doses of bisphosphonates can lead to fetal abnormality of the skeleton and the kidney (273).

B. Human adverse events

As in animals, studies in humans have revealed only a few significant adverse events. Caution must be taken with all intravenous administrations of large amounts of bisphosphonates since rapid injection has led to renal failure (269), probably because the bisphosphonate is forming a solid phase in the blood, which is then retained in the kidney. No such events have occurred since care is taken to administer all bisphosphonates in large amounts by slow infusion in plenty of fluids.

The oral administration of bisphosphonates, especially those with a primary amine, can be accompanied by esophageal and gastrointestinal side effects such as nausea, dyspepsia, vomiting, gastric pain, and diarrhea, and sometimes even ulceration (129, 130, 274). These adverse events have decreased since patients began ingesting the drug with adequate water and without reclining after its intake to minimize esophageal reflux.

As seen in animals, etidronate, when given at daily oral doses of 400–800 mg, can produce an inhibition of normal skeletal mineralization, leading to a clinical and histological picture of osteomalacia. This condition regresses after discontinuation of therapy (31, 49, 50). Similar results have been seen with pamidronate in Paget's disease when given intravenously at doses equal to or higher than 180 mg per year (51, 52).

The last commonly seen effect, which has been mentioned earlier in this paper, is observed after intravenous administration of more potent bisphosphonates containing a nitrogen atom. This is not observed with etidronate, clodronate, or tiludronate. After intravenous administration, a transient pyrexia of usually 1–2°C, sometimes more, accompanied by flu-like symptoms, may occur. It is maximal within 24–48 h and disappears after approximately 3 days, in spite of continued treatment. It is usually observed only once, even if treatment is continued and restarted later (213). The mechanism of these changes, which resemble an acute phase response, seems to involve the stimulation of macrophages to release IL-6 and TNFα (215, 216), both of which increase in plasma.

Most of the other adverse events are seen only occasionally, and it is not certain to what extent they are actually related to the drugs.

VII. Conclusion

Since the discovery of their effects on biological tissues in 1968, much progress has been made in our understanding of the mechanisms of action of the bisphosphonates. While the effects on mineralization appear to be physicochemical by inhibiting crystal growth, those on resorption are cellular. However, we still do not know the molecular mechanisms leading to the inhibition of resorption. The consensus is that the final effect is through the osteoclasts, but we do not know how much is via the inhibition of their activity and how much is due to a decrease in their number. It is also unknown how much of the effect is direct or indirect through other cells, such as the osteoblasts. It is agreed that the bisphosphonates need the P-C-P bond to target themselves to the mineral; however, the effect on cells occurs in part even when no mineral is present while they are exposed to the drug. Thus, the cells may be modulated by the bisphosphonate liberated from the mineral, their potency being determined by the structure of the lateral chain. Finally, we have practically no knowledge as to which part of the molecule is responsible for the effect, nor what the optimal structure of a compound for this effect is. The latter is regrettable since such knowledge would not only allow us to synthesize new and better inhibitors, but also give us an insight into the mechanisms of bone resorption in general. Further research in this direction is therefore desirable.

Current clinical applications for the inhibition of bone resorption are Paget's disease, tumor bone disease, and osteoporosis. Future applications could be, among others, Sudeck's atrophy, fibrous dysplasia, loosening of bone implants, and alveolar resorption. As to their property of inhibiting calcification, only etidronate is currently used with variable success for ectopic calcification and ossification.

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